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UNIVERSITY OF ALBERTA

SYNTHETIC PEPTIDE STUDIES ON TROPONIN C-TROPONIN I INTERACTION

by

NGAI, SAI MING ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Synthetic Peptide Studies on TnC-TnI Interaction" submitted by Sai Ming Ngai in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The interaction between troponin I (TnI) and troponin C (TnC) plays a critical role in the regulation of muscle contraction. In this thesis, synthetic peptide chemistry is used to investigate the molecular mechanism involved in the TnI/TnC interaction that governs the regulation of muscle contraction.

The troponin I (TnI) inhibitory region (104-115) is synthesized with Gly-104 labeled [α-^{14}C] and a covalently linked benzoylbenzoyl (BB-) moiety at the N-terminus to yield a photoactivatable radioactive peptide (BBIp). BBIp is crosslinked to rabbit skeletal muscle troponin C (TnC) to locate the Ip binding site on TnC. From the cross-linking results, we have generated a three-dimensional model of the TnC C-domain/Ip complex using molecular dynamic and energy minimization calculations. Other investigations demonstrate that the TnI N-terminal peptide (TnI1-40) is able to prevent TnI or Ip from interacting with TnC. Subsequently, it has been shown that a shorter TnI peptide (TnI1-30) retains the properties of TnI1-40.

To further characterize the interaction between TnI1-30 and TnC, TnC mutants that carry a single tryptophan fluorescence probe in either the N- or C-domain [F105W (1-162), F105W/C domain (88-162), F29W (1-162) and F29W/N domain (1-90)] are employed. It was demonstrated that the major TnI1-30 binding site on TnC is located on the C-domain of TnC. Low Kd values (Kd < 10^{-7} M) are obtained from the interaction of F105W (1-162) and F105W/C domain (88-162) with TnI1-30.

Finally, TnI peptides [N-terminal peptides (TnI1-40 and TnI1-30) and inhibitory peptide/Ip (TnI104-115)], recombinant TnC and TnI mutants are used to investigate the structural and functional relationship between TnI and TnC. Our results indicate that the N- and C-terminal regions of TnI that flank the inhibitory region, residues 104 to 115

play a vital role in modulating the Ca^{2+}- sensitive release of the TnI inhibitory region (residues 104-115) by TnC within the muscle filament. We conclude that the Ca^{2+}-dependent regulatory process for the TnI/TnC interaction is more complex than previously thought.

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TABLE OF CONTENTS

Page

CHAPTER I: Introduction 1

THE THICK FILAMENT 4

THE THIN FILAMENT 4

INTERACTIONS OF THIN FILAMENT REGULATORY PROTEINS 18

MODELS OF MUSCLE REGULATION 25

PURPOSE OF THIS STUDY 32

References 34

CHAPTER II: Materials and Methods 44

PEPTIDE SYNTHESIS AND PURIFICATION 44

PROTEIN PURIFICATION 46

POLYACRYLAMIDE ELECTROPHORESIS (PAGE) 47

ATPase ASSAYS 49

CENTRIFUGATION STUDY 50

SIZE EXCLUSION CHROMATOGRAPHY 51

CROSSLINKING STUDIES 52

TRYPTIC DIGESTION OF TNC-BBIP COMPLEX 54

CYANOGEN BROMIDE CLEAVAGE 54

AMINO ACID SEQUENCING AND MASS SPECTROMETRY 54

COMPUTER MODELING 55

FLUORESCENCE SPECTROSCOPY 56

CIRCULAR DICHROISM SPECTROSCOPY 56

References.. 58

CHAPTER III: Photochemical Crosslinking between Native Rabbit Skeletal Troponin C and Benzoylbenzoyl Troponin I Inhibitory Peptide, Residues 104-115

ABSTRACT.. 60
INTRODUCTION.. 60
RESULTS.. 61
Photolysis of TnC and BBIp 61
Acto-S1-TM ATPase Assay 65
Identification of the site of crosslinking.. 65
Three Dimensional Computer Modeling.. 71
DISCUSSION.. 74
REFERENCES.. 81

CHAPTER IV: Biologically Important Interactions between Synthetic Peptides of the N-terminal Region of TnI and TnC

ABSTRACT.. 84
INTRODUCTION.. 84
RESULTS.. 86
Effect of TnI N-terminal Regulatory Peptide 1-40 (Rp) and Truncated TnI Analogs, TnI (10-40) and TnI (20-40) on the Acto-S1-TM ATPase Activity 86

Effect of Rp and Truncated TnI Analogs, TnI(10-40) and TnI(20-40) on TnC Release (in the Presence and Absence of Ca^{2+}) of the Acto-S1-TM ATPase Inhibition by TnI or TnI Inhibitory Peptide (Ip) 86

Determination of the Interaction Between TnI, TnC and Rp by Polyacrylamide Gel Electrophoresis 90

Study of the Interaction between Rp and TnC by Size Exclusion Chromatography 94

DISCUSSION 98

REFERENCES 102

CHAPTER V: Characterization of the Biologically Important Interaction between Troponin C and the N-terminal Region of Troponin I

ABSTRACT 104

INTRODUCTION 105

RESULTS AND DISCUSSION 105

Effect of TnI N-terminal Peptides on the ability of TnC (+Ca^{2+}) to Release Ip (104-115) Inhibition of Acto-S1-TM ATPase 105

Effect of BBRp on the ability of TnC (+Ca^{2+}) to Release Ip (104-115) Inhibition of Acto-S1-TM ATPase 112

Interaction of TnC mutants with TnI1-30 112

Complex Formation between TnC and TnI N-terminal Peptide Before and After Photolysis 112

Impact of TnI1-30 on the fluorescence property of TnC mutants [F105W, F105W/C domain (88-162), F29W and F29W/N domain (1-90)] 117

Fluorescence titration of the TnC mutants [F105W and F105W/C domain (88-162)] against TnI1-30 117

REFERENCES 124

CHAPTER VI: Structural and Functional Studies on Troponin I and Troponin C Interactions

ABSTRACT 125

INTRODUCTION 125

RESULTS 126

Effect of TnI1-30 on recombinant TnC mutants in release of the Acto-S1-TM ATPase Inhibition by TnI Inhibitory Peptide ((p) 126

Study of the Interaction between TnI1-30 and TnC mutants by Size Exclusion (SEC) and reversed-phase Liquid Chromatography (RPC) 129

Ca^{2+} Titration Profile of Rabbit Skeletal TnC and the TnC-TnI1-30 Complex as monitored by far-UV Circular dichroism 132

Effect of TnI1-30 on the Interaction between Recombinant TnC and TnI mutants 132

DISCUSSION 140

REFERENCES 146

CHAPTER VII: Discussion and Future Research 149

REFERENCES.. 156

LIST OF TABLES

Page

Table I-1 Properties of various muscle proteins.................................. 3

Table I-2 Sites of Interaction between Muscle Proteins........................... 7

Table III-1 Amino Acid Sequences of TnI and TnC Peptides...................... 62

Table III-2 Sequence Analysis of the Radioactive Fraction......................... 69

Table III-3 Solvent accessible area of residues in TnC, Ip or TnC/Ip complex... 75

Table III-4 Summary of Solvent Accessible Surface Areas (ASA , Å^2).......... 76

Table IV-1 Amino Acid Sequences of TnI Peptides................................ 85

Table IV-2 Centrifugation Studies of Ip or Rp Binding With Actin-TM.......... 93

Table V-1 Amino Acid Sequences of TnI Peptides................................ 106

Table VI-1 Amino Acid Sequences of TnI Peptides................................ 127

Table VI-2 Effect of TnI Regulatory Peptide (TnI1-30) on the pKd Values and Hill Coefficients for the Low- and High-Affinity Ca^{2+} Binding Sites of the Skeletal TnC....................................... 134

LIST OF FIGURES

Page

Fig. I-1. The sliding filament hypothesis of the mechanism of muscle contraction.......... 2

Fig. I-2. The myosin molecule.......... 5

Fig. I-3. A model for the molecular arrangement of the thin filament proteins.......... 6

Fig. I-4. The X-ray crystal structure of G-actin derived from the Actin : DNAse 1 complex.......... 10

Fig. I-5. Coiled coil structure of TM.......... 11

Fig. I-6. The X-ray crystal structure of turkey skeletal troponin C.......... 13

Fig. I-7. Schematic diagram showing the dominant protein interactions involved in the regulation of muscle contraction.......... 24

Fig. I-8. Schematic description of the sliding-filament-cross-bridge theory.......... 26

Fig. I-9 Cross section of skeletal muscle thin and thick filaments illustrating possible change of interactions upon the binding of Ca^{2+} to TnC.......... 28

Fig. I-10. Simplistic scheme for the coupling of ATP hydrolysis cycle to kinetic steps involved during muscle contraction.......... 29

Fig. I-11. Currently popular "modified-refractory-state model" relating cross-bridge cycling to ATP splitting.......... 31

Fig. II-1 DEAE-52 Cellulose purification of rabbit skeletal S1.......... 48

Fig. II-2 Schematic diagram of a photoaffinity labile peptide and its target protein.......... 53

Fig. III-1. Photolysis Experiment.......... 63

Fig. III-2. Effect of Ip, BBIp, TnC, TnC/BBIp and TnC-BBIp on the acto-S1-TM ATPase Activity.................................. 66

Fig. III-3. Complete Tryptic Digest of TnC/BBIp complex...................... 67

Fig. III-4. Summary for Characterization of the TnC/BBIp Complex.......... 70

Fig. III-5. Molecular modeling.. 72

FIG. IV-1. Effect of TnI and TnI peptides on the acto-S1-TM ATPase Activity... 87

FIG. IV-2. Effect of N-terminal TnI peptide on TnC (Ca^{2+}) in release of acto-S1-TM ATPase inhibition by TnI or TnI inhibitory peptide (Ip)... 88

FIG. IV-3. Effect of Rp (TnI 1-40) on the release of acto-S1-TM inhibition by TnC in the absence or presence of Ca^{2+}............... 91

FIG. IV-4. Polyacrylamide gel electrophoresis.................................... 95

FIG. IV-5. Study of the interaction between Rp and TnC by HPLC............ 96

Fig. V-1. TnC mutants (F105W, F105W/C domain (88-162), F29W and F29W/N domain (1-90)).................................. 107

Fig. V-2. Effect of the N-terminal TnI peptide on the ability of TnC (Ca^{2+}) to release the acto-S1-TM ATPase inhibition by TnI inhibitory peptide (Ip).. 110

Fig. V-3. Polyacrylamide gel electrophoresis.................................... 113

Fig. V-4. Fluorescence Spectra.. 118

Fig. V-5. Fluorescence Titration.. 119

Fig. V-6. Determination of the Binding Constant for F105W and F105W/C domain (88-162).. 121

Fig. VI-1. Effect of N-terminal TnI peptide on TnC mutant (Ca^{2+}) in release of acto-S1-TM ATPase inhibition by TnI inhibitory peptide (Ip).. 128

Fig. VI-2. Study of the interaction between TnI1-30 and TnC mutant by HPLC 130
Fig. VI-3. Effect of TnI regulatory peptide, TnI1-30 on Ca^{2+} binding to skeletal TnC monitored by circular dichroism 133
Fig. VI-4. Polyacrylamide gel electrophoresis 136
Fig. VI-5. Summary for TnI/TnC Interaction 144
Fig. VII-1. Structure of photoafinity probes used to prepare synthetic probe-peptides 151
Fig. VII-2. Schematic diagram showing the dominant protein interactions involved in the regulation of muscle contraction 154

ABBREVIATION

A1/A2 light chains,	low molecular weight polypeptide associated with myosin S1 head that can be removed from S1 heavy chain under alkali condition
Ac-,	acetylated terminus
actin-TnI,	actin and troponin I complex
acto-S1,	actin and myosin subfragment 1 complex
-Amide,	amidated C-terminus
BB,	benzoylbenzoyl moiety
BBIp,	N α-Benzoylbenzoyl TnI (104-115) amide
BBRp,	N α-benzoylbenzoyl TnI (1-40 residues) amide
Boc,	N-tert-butyloxycarbonyl
CaM,	calmodulin
CD,	circular dichroism
-COOH,	free carboxyl terminus
DCC,	dicyclohexylcarbodiimide
DCM,	dichloromethane
DIEA,	diisopropylethylamine
DMF,	dimethylformamide
DTNB light chains,	small molecular weight polypeptides associated with myosin S1 head which can be removed from S1 heavy chain with treatment of DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]
DTT,	dithiothreitol
EDC,	1-ethyl-3-[3-(dimethlamino) propyl]carbodiimide
EDTA,	ethylene-diaminetetra-acetic acid
EGTA,	ethylene glycol bis(β-amino ethyl ether) N,N,N',N'-tetra-acetic acid
F-actin,	fibrous actin protein
G-actin,	globular actin protein
HMM,	heavy meromyosin
HPLC,	high-performance liquid chromatography
Ip,	TnI inhibitory peptide Ac-TnI (104-115) amide
Ip-^{14}C,	radioactive TnI inhibitory peptide (104-115)
LMM,	light meromyosin
MLCK peptide,	myosin light chain kinase peptide (residues 1-20)
MOPS,	3-(4-Morpholino) propane sulfonic acid
Mr,	molecular weight

NH_2-,	free amino terminus
NMR,	nuclear magnetic resonance
pCa	- log $[Ca^{2+}]$
Rp,	TnI N-terminal regulatory peptide (1-40)
RPC,	reversed-phase chromatography
S1,	myosin subfragment 1
SEC,	size-exclusion chromatography
TFA,	trifluoroacetic
TM,	tropomyosin
Tn,	troponin
TnC,	troponin C
TnI,	troponin I
TnT,	troponin T
Tris,	tris-(hydroxymethyl) amino methane

CHAPTER I

INTRODUCTION

Contractile movement has always been associated with life and the elucidation of the molecular mechanism of muscle contraction has been a subject of interest for decades. The ultimate understanding of muscle contraction will advance our knowledge on the metabolic control and electrical processes as well as the complex protein structural arrangement. There are three kinds of muscle, skeletal (voluntary) muscle, cardiac muscle and smooth muscle. Skeletal and cardiac muscles are cross-striated and there are large similarities between these tissues. A basic outline of muscle contraction in vertebrate skeletal muscle can be found in many biochemistry textbooks (such as, Stryer, 1988 and Voet and Voet, 1990) and various aspects of regulation of muscle contraction have been previously reviewed (Da Silva and Reinach, 1990; Eisenberg and Hill, 1985; El-Saleh *et al.*, 1986; Leavis and Gergely, 1984; Ohtsuki *etal.*, 1986; Phillips *etal.*, 1986; Strynadka and James, 1991; Żot and Potter, 1987).

With the development of modern technology in the mid-1950s, clues to the structure and function of different muscle protein components have been determined from their ultrastructure using both light and electron microscopy and X-ray analysis of the corresponding paracrystals. Major muscle proteins involved in the contraction and relaxation process can be isolated and studied through standard biochemical procedures, such as amino acid sequencing and analysis, nuclear magnetic resonance (NMR), assaying of enzymatic activity, circular dichroism (CD), ultra-centrifugation and others. The basic structural organization of striated muscle tissue consists of two types of filaments, thick and thin, which are interdigitated and slide past one another when muscle contracts (Fig. I-1) (Draper and Hodge, 1949; Huxley, H. E., 1953; Rosa *etal.*, 1950). Table I-1 lists the properties of the major proteins that are present in the thick and thin filaments.

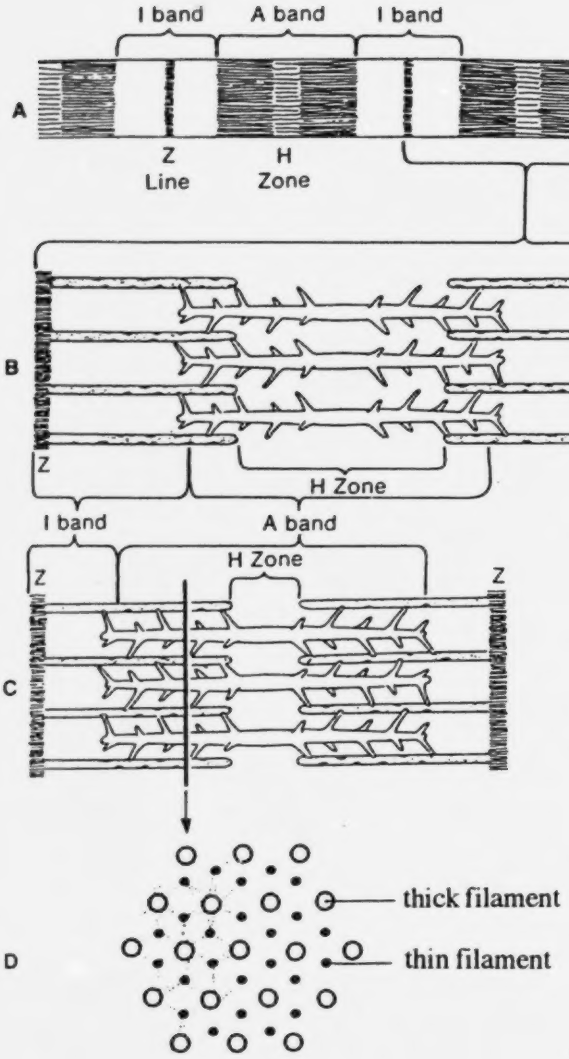
I band
A band
I band
A
Z
Line
H
Zone
B
Z
H Zone
Z
I band
A band
H Zone
Z
Z
C
thick filament
D
thin filament

Fig. I-1. **The sliding filament hypothesis of the mechanism of muscle contraction**. A, Diagram of part of a single myofibril showing the pattern of light (I) and dark (A) bands. B, Longitudinal view of the arrangement of thick and thin filaments within a myofibril in the relaxed state. C, Longitudinal view of the arrangement of thick and thin filaments in a contracted myofibril, showing that the I band decreases in thickness. Note that the two types of filaments slide past one another during contraction. D, Transverse view through C at arrow, showing each thick primary filament surrounded by six thinner secondary filaments. (Figure taken from Huxley, H.E., 1969.)

Table I-1 Properties of various muscle proteins

Protein	Mr (Daltons)	Number of Residues	Characteristic	Reference
Myosin	470,000		ATPase activity	Lowey and Cohen, 1962
S1	95,000		ATPase activity	Weeds and Taylor, 1975
Actin	42,000	375	activates ATPase	Elzinga *et al.*, 1973
α-TM	33,000 (monomer)	284 (monomer)	binds actin and TnT	Stone *et al.*, 1974
Skeletal TnC	17,846	159	binds four Ca^{2+}	Collins *et al.*, 1977
Skeletal TnI	20,864	178	inhibits ATPase	Wilkinson and Grand, 1975, 1978
Skeletal TnT	30,503	259	binds TM	Pearlstone and Smillie, 1976

THE THICK FILAMENT

The thick filament is composed of the long match stick-like myosin molecules (Fig. I-1) which are organized into a bipolar structure in which the tails form the core of the filament and the heads projecting out at both ends. Myosin is an enzyme capable of hydrolyzing ATP (ATPase activity) and is composed of two heavy chains and four non-covalently associated light chains. The carboxyl terminal region of the two heavy chains forms a double stranded α-helical coiled coil whereas the two amino termini fold into two globular heads. The ATPase activity and the actin binding properties are located in these two heads. Myosin molecules can be specifically cleaved into fragments or sub-fragments through exposure to certain enzymes (Fig. I-2). Trypsin and papain can cleave the myosin rod partway along its length, leaving two fractions known as light meromyosin (LMM) and heavy meromyosin (HMM). Chymotrypsin can cleave the myosin and yield a single-headed myosin sub-fragment (S1). Both S1 and HMM retain the ATPase activity and the ability to bind actin and they are soluble under physiological ionic strength. S1 and HMM are often used in place of myosin in the ATPase assay. The two DTNB light chains which are not required for ATPase activity are destroyed during the enzymatic digestion with chymotrypsin in preparing S1. The other two light chains A1 (M.W. 21,000) and A2 (M.W. 16,200) are essential for ATPase activity and remain associated with the globular heads after digestion.

THE THIN FILAMENT

The thin filament (Fig. I-3) is made up of three major proteins which are actin, tropomyosin (TM), and troponin (Tn). Table I-2 lists the corresponding protein-protein interactions found within the thin filament.

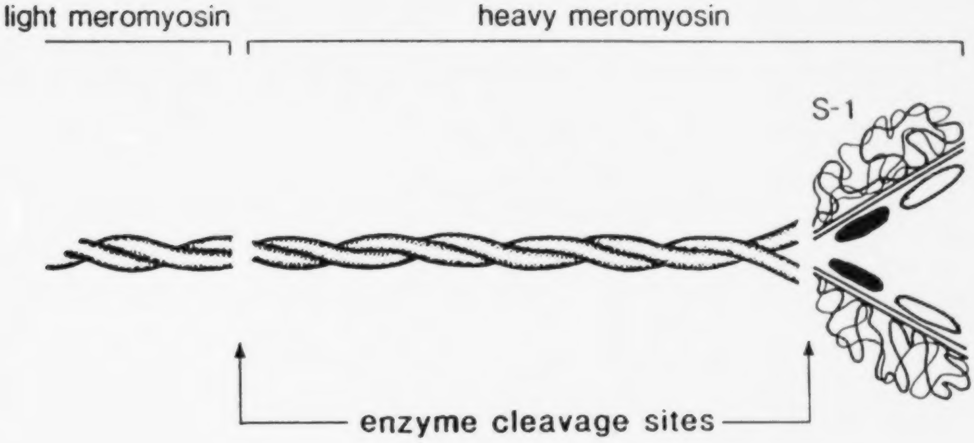
light meromyosin
heavy meromyosin
S-1
enzyme cleavage sites

Fig. I-2. **The myosin molecule, shown schematically**. Fragment and subfragment nomenclature is given. Also shown are cleavage sites of several enzymes. The molecule is composed of six polypeptide chains : two identical heavy chains and two pairs of light chains. Heads at right, rod (tail) at left.

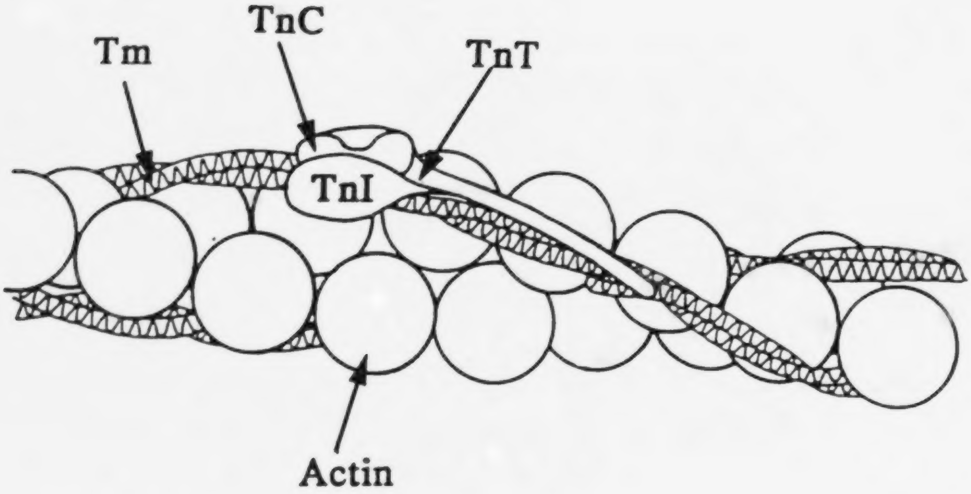

Fig. I-3. **A model for the molecular arrangement of the thin filament proteins**. Two strands of F-actin molecules (open circles) form the core of the structure. Tropomyosin (TM) molecules are shown as coiled-coils which lie in the grooves of the actin filament. Each TM molecule spans 7 G-actin monomers on each of the two strands of the actin filament and interacts with one troponin (Tn) complex. The troponin complex (TnI, TnT, and TnC) is positioned at regular intervals (385 Å) on the thin filament such that there is a molar ratio of G-actin to TM to Tn of 7:1:1. The Tn-TM complex form the basic regulatory unit of the thin filament (Figure taken from Heeley *et al.*, 1987).

Table I-2
Sites of Interaction between Muscle Proteins

	Putative Binding Region (amino acid residues)					
	Actin	TM	TnT	TnI	TnC	S1
Actin				(96-116)[13] (104-115)[33,37]		A1 light chain [28,29] (702-708) [30] 20 and 50 kDa junction [16,29,40]
TM	95, 61 [1,10] (70-86) [11] (340-365) [11]		(71-151) [22] (159-259) [24] (197-259) [21,22,25,34]	(96-116) [31,5] (104-115) [19,33,17]		(237-335) [32]
TnT		(190-284) [3,14,22]		(40-78) [9] (1-57) [27]	(1-100) [6,8,22,35,39] (121-159) [12,20]	
TnI	(1-28) [38] (1-12) [7] (1-44) [13]		(159-259) [9,15,26]		(50-60) [6,12] (89-100) [4,6,12,39] (126-136) [6]	
TnC			(159-259) [21,23] (159-227) [21,23] (176-230) [15,21,23]	(1-40) [17] (1-30) [18] (1-47) [31] (104-115) [19,31,33,37] (96-116) [4,31]		
S1	(1-12) [28] (1-28) [38] (40-113) [2] (361-364) [36]					

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37. Van Eyk and Hodges, 1988.
38. Van Eyk and Hodges, 1991a.
39. Weeks and Perry, 1978.
40. Yamamoto and Sekine, 1979.

I. Actin

Actin is a highly conserved protein (Vanderkerckhove and Weber, 1978) and forms the major constituent of thin filaments. In solution of low ionic strength, actin is globular protein (G-actin) with the size of 42 kDalton. As the ionic strength is increased to the physiologic level (2 mM Mg^{2+}, 50 mM KCl), G-actin polymerizes into a fibrous form called F-actin. In muscle, F-actin consists of two strands coiled about one another. Recently, the x-ray crystal structure of a G-actin/DNAase complex has been determined (Kabsch *et al.*, 1990; see Fig. I-4).

II. Tropomyosin (TM)

TM exists as a dimer of two polypeptide chains. Two types of TM subunits are found in skeletal muscle, α and β (Cummins and Perry, 1973). The proportions of each subunit type appear to be tissue specific (Cummins and Perry, 1974; Dhoot and Perry, 1979). Each subunit (α or β) consists of 284 amino acid residues with a molecular weight of 33 kDaltons. The α and β subunits have similar amino acid sequences (Sodek *et al.*, 1972; Stone et al., 1975; Stone and Smillie, 1978 and Mak *et al.*, 1980). The two TM polypeptide chains associate in register in a coiled-coil fashion (Crick, 1953; Hodges *et al.*, 1972; Sodek *et al.*, 1972; Johnson and Smillie, 1975 and O'Shea *et al.*, 1991). In rabbit skeletal tropomyosin, dimers consisting of two α-subunits are most common. Throughout the entire 284-residue sequence of each chain, a regular pattern of repeating heptads (4-3 or 3-4 hydrophobic repeat) in which nonpolar residues are found at positions a and d (Hodges *et al.*, 1972; Sodek *et al.*, 1972; McLachlan and Stewart, 1975) (Fig. I-5). Furthermore, a high proportion of basic amino acids occurs in position g and acidic amino acids in position e. Since the two helical chains run in the same direction with 3.6 residues per turn, nonpolar residues from one chain face non-polar residues from the other in the surface of contact between the two. The basic residues in position g of one chain line up with acidic

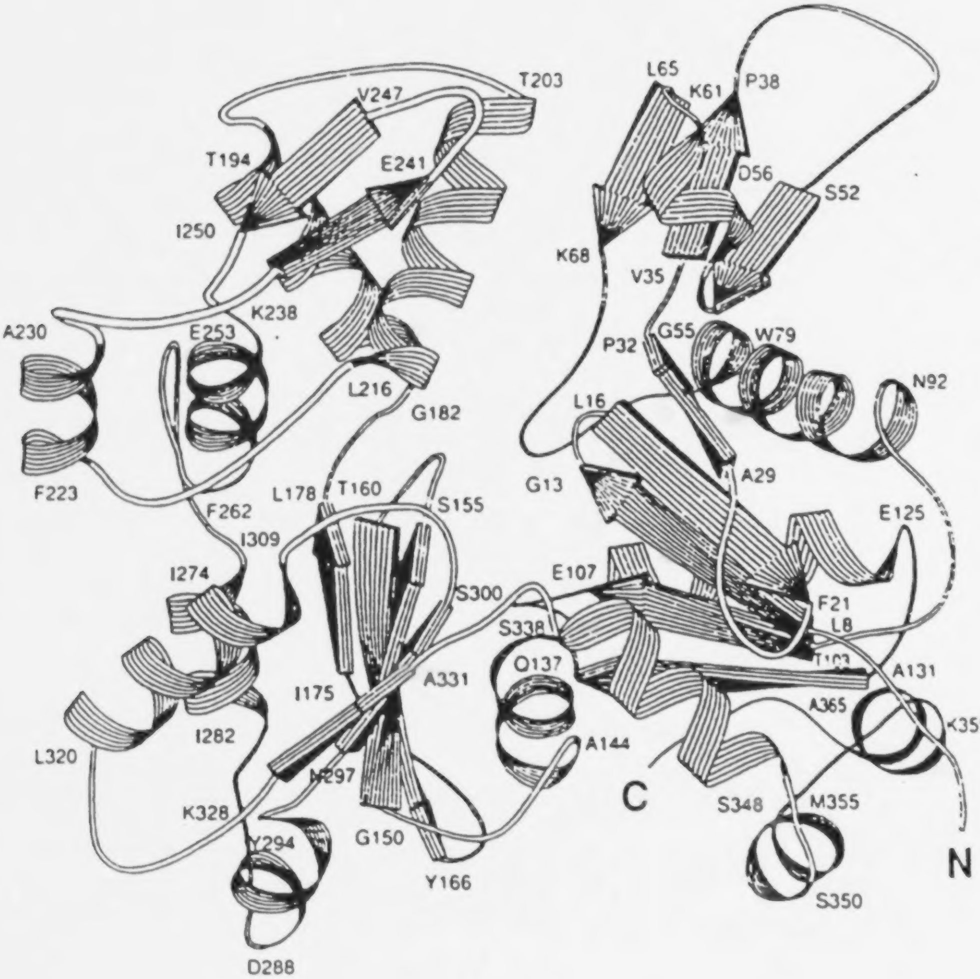
T203
V247
L65
K61
P38
T194
E241
D56
S52
I250
K68
V35
K238
A230
E253
G55
W79
P32
N92
L216
G182
L16
A29
F223
L178
T160
S155
G13
F262
E125
I309
E107
I274
S300
F21
L8
S338
T103
A131
Q137
A331
I175
A365
K359
L320
I282
A144
N297
C
S348
M355
K328
Y294
G150
N
Y166
S350
D288

Fig. I-4. **The X-ray crystal structure of G-actin derived from the Actin : DNAse 1 complex**. The determined three dimensional structure of the G-actin is shown as a ribbon diagram (Figure taken from Kabsch *et al.*, 1990). Amino acid residues are labeled periodically throughout the molecule.

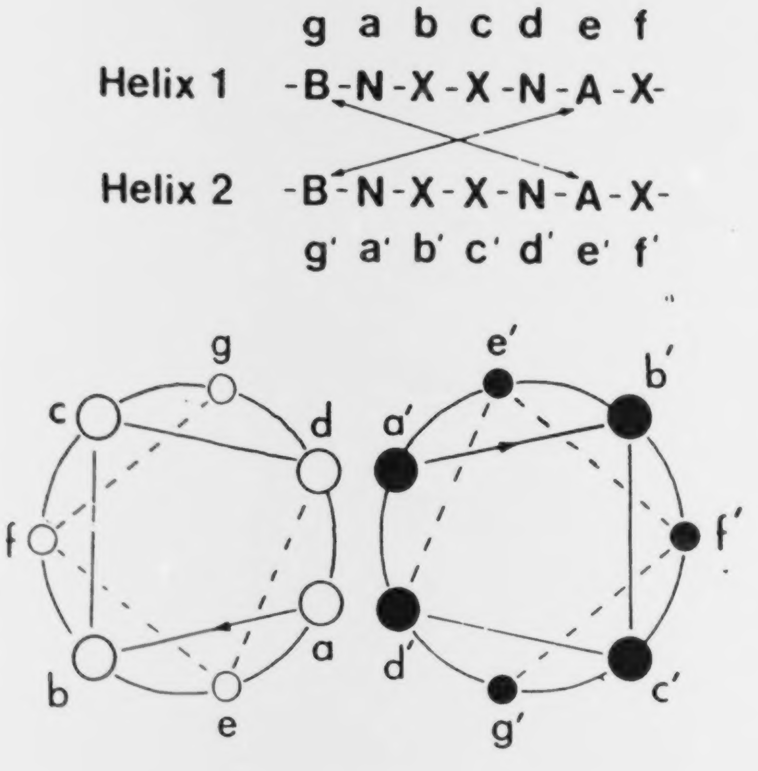

Fig. I-5. **Coiled coil structure of TM**. Top: The heptad repeat in tropomyosin. The seven amino acid residues are labelled a to g with hydrophobic residues (N) at positions a and d and a high proportion of basic (B) and acidic (A) residues at positions g and e, respectively. The possible electrostatic interactions that can occur between the g and e positions of the two helices of the coiled coil are indicated by the double-headed arrows. Bottom: Cross-sectional representation of the two coiled coil helices looking from the N-terminus. The coiled coil structure is stabilized by interactions of hydrophobic residues in positions a and a' and d and d'. Electrostatic interactions occur between g and e' and e and g' (Figure taken from Smillie, L. B., 1979).

residues in position e of proceeding heptad in the other chain leading to stabilizing electrostatic interactions (Hodges *et al.*, 1972; Sodek *et al.*, 1975; McLachlan and Stewart, 1975; Hodges *et al.*, 1981). The overall structure corresponds to the classic "knobs and hole" type of alignment proposed in 1953 by Crick for coiled-coil structures in general. Head-to-tail overlap of tropomyosin coiled-coils in the thin filament appears to occur over eight or nine residues at the N- and C-terminus of the molecule. The interaction of these residues is strengthened by hydrophobic and electrostatic interactions (Johnson and Smillie, 1975; Mak *et al.*, 1981; Mak and Smillie, 1983).

III. Troponin (Tn)

Tn is a complex of three proteins: troponin T (TnT), the tropomyosin binding protein which binds the troponin complex to tropomyosin, troponin C (TnC), the calcium binding protein and troponin I (TnI), the inhibitory protein which inhibits actomyosin ATPase activity. The Tn complex is positioned at regular intervals (385 Å) on the actin filament such that there is a molar ratio of G-actin to TM to Tn of 7:1:1.

Troponin C (TnC) Rabbit skeletal TnC is composed of 159 residues with a calculated molecular weight of 17,965 . TnC is a highly conserved molecule among types and species (Collins *et al.*, 1973, 1977). It is an acidic protein [pI values in the range of 4.1 to 4.4 (Hartshorne and Driezen, 1972)] containing high content of glutamic and aspartic acids. The x-ray structures of chicken (Sundaralingam *et al.*, 1985) and turkey (Herzberg and James, 1985) skeletal TnC have been determined. TnC is a dumbbell-shaped molecule, approximately 75 Å long, with distinct N-terminal and C-terminal globular domains linked via a long nine-turn α-helix (Fig. I-6). Each domain of the TnC contains two Ca^{2+}-binding loops with the EF hand helix-loop-helix structural motif (Kretsinger and Nockolds, 1973; Collins *et al.*, 1973; Vanaman *et al.*, 1977). In skeletal TnC, the high affinity sites (sites III and IV) located in the C-terminal domain bind both Ca^{2+} and Mg^{2+} with association

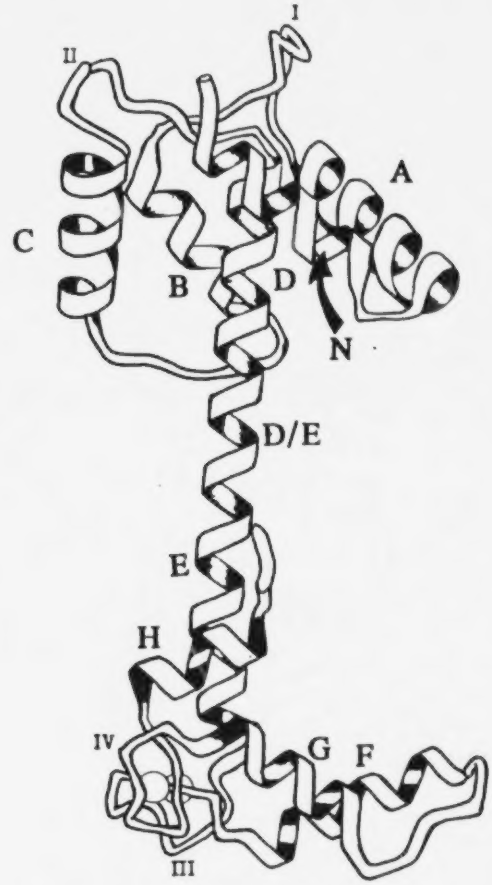

Fig. I-6. **The X-ray crystal structure of turkey skeletal troponin C**. It is shown as a ribbon diagram (Figure taken from Herzberg and James, 1985). The N-terminal domain (top) is separated from the C-terminal domain (bottom) by a long central α-helix. The low affinity Ca^{2+} specific sites I and II located in the N-terminal domain are unoccupied in this crystal form. The high affinity Ca^{2+}/Mg^{2+} sites III and IV in the C-terminal domain are filled with Ca^{2+}. The helices involved in the Ca^{2+} binding sites are labeled A to H sequentially with the additional helix in the N-terminus of the molecule labeled as the N-helix.

constants of 2×10^7 and $5 \times 10^3 M^{-1}$, respectively. The low affinity sites (sites I and II) located in the N-terminal domain can bind Ca^{2+} with a lower association constant of $2 \times 10^5 M^{-1}$ (Potter and Gergely, 1974).

The low affinity sites (sites I and II) in the N-terminal domain of TnC have been implicated as the relevant triggering sites that initiate muscle contraction (Johnson *et al.*, 1979; Robertson *et al.*, 1981). At resting levels of Ca^{2+} (pCa ~ 7-8) only the high affinity sites (sites III and IV) of TnC are filled. Upon nerve stimulation and followed by the subsequent increase in sarcoplasmic Ca^{2+} concentration (pCa ~ 4-5), the low affinity sites (sites I and II) become filled. The conformational change associated with Ca^{2+} binding to the low affinity Ca^{2+} binding sites (sites I and II) of TnC is believed to be the signal that initiates muscle contraction. In the crystal structure (Fig. I-6), the Ca^{2+} binding sites I and II are in the Ca^{2+}-free state. The apo state seems to be stabilized due to the interaction between the various helices comprising the Ca^{2+} binding sites I and II and the N-helix (residues 3-13). There is no counterpart to the N-helix in the C-terminal domain. This suggests that the Ca^{2+} affinity with the low affinity sites may not only be a function of the residues within the Ca^{2+} coordinating loop, but also a function of the tertiary folding of this domain. The homology between the calmodulin (CaM) and TnC primary sequences are reflected in the large degree of similarity in the tertiary structure of the proteins. Both molecules have two major folded N- and C-domains, each containing two Ca^{2+}-binding sites separated by a long central helix (Herzberg and James, 1985; Sundaralingam *et al.*, 1985; Babu *et al.*, 1985). However, CaM has no N helix in the N-domain and it has been concluded that CaM has four equivalent Ca^{2+}-binding sites, which compete for both Mg^{2+} and Ca^{2+} (Ilda and Potter, 1986). Studies using recombinant N and C-domains of TnC indicate a summation of the far UV CD spectra ($\pm Ca^{2+}$) of the two domains was virtually superimposible on that of the intact TnC protein and data also suggest a significant Ca^{2+}-induced transition involving secondary structural elements of the N-domain (about 27% of the total Ca^{2+}-induced ellipticity) (Li *et al.*, 1994). Other studies on synthetic single-site-peptides (which carry a single Ca^{2+}-binding loop of TnC) indicate that a two-site unit (dimer of two single-site-peptide) represents a structural domain. There is little evidence of any interaction found between the two domains of TnC (Shaw *et al.*, 1990, 1991; Kay *et al.*, 1991).

Although the centers of the two domains of TnC are widely separated (about 40 Å) as shown in the x-ray structure (Fig. I-6), it may be possible that any conformational change that occurs upon Ca^{2+} binding at either site I or II of the N-domain of TnC may be transmitted to the C-domain of TnC via the central D/E helix. Wang *et al.* (1990) demonstrated that binding of cations to the high affinity sites (sites III and IV) of a mutant TnC altered the environment around the amino acid at position 57 in the N-terminal domain (sites I and II). Grabarek *et al.* (1986) and Rosenfeld and Taylor (1985) showed that the binding of Ca^{2+} to the low affinity sites altered the environment around Cys 98 in the C-terminal domain. Nevertheless, it is also possible that the signal is transmitted through other Tn proteins, TnI and TnT. Based on computer modeling of the x-ray crystal structure of TnC, Herzberg *et al.* (1986) predicted that residues in the C-helix would become more exposed upon Ca^{2+} binding to the low affinity sites. The hydrophobic cavity exposed in the Ca^{2+}-bound form is thought to provide an interaction site with TnI (Strynadka and James, 1989). Present Ca^{2+} titration studies on the N-domain of TnC monitored by NMR methodology demonstrates reasonable agreement with the above hypothesis (Tsuda *et al.*, personal communication).These findings indicated that the N and C-terminal domains of TnC may communicate with each other upon the binding of Ca^{2+} or Mg^{2+}.

Troponin I (TnI) Functionally, TnI is the inhibitory component of the Tn complex in regulation of muscle contraction (Greaser and Gergely, 1971, 1973). Rabbit skeletal TnI is composed of 181 amino acid residues and has a calculated molecular weight of 21,162. The TnI protein has 36 acidic and 40 basic amino acid residues that account for the high pI value of 9.3 (Wilkinson, 1974). There is one tyrosine residue at position 43, a single tryptophan at position 160, and three cysteine residues at positions 48, 64 and 133 in the primary sequence (Wilkinson and Grand, 1975; Sheng *et al.* 1992). Oxidation state of the three cysteine residues is related to the ability of TnI to form a functional complex with other troponin subunits (Horwitz *et al.*, 1979; Hincke *et al.*, 1979). The maintenance of the sulfhydryl groups of TnI in the reduced state is essential in order to demonstrate full biological activity and interaction between other Tn proteins. Two residues of isolated skeletal TnI can be phosphorylated: Thr 11 by phosphorylase kinase and Ser 118 by cardiac 3', 5' -cyclic AMP-dependent protein kinase isolated from bovine cardiac muscle (Huang *et al.*, 1974; Moir *et al.*, 1977). The rate of phosphorylation at both sites of TnI (Thr 11 and Ser 118) can be reduced by formation of the TnI/TnC complex and addition of Ca^{2+} to the TnI/TnC complex further inhibits the phosphorylation of Thr 11, although formation of the complex using previously phosphorylated TnI is not hindered. This suggests that skeletal TnI may not be phosphorylated in the whole Tn complex, and no structural or functional role has yet been established for skeletal TnI phosphorylation.

Until now, there is no direct information on the three-dimensional structure of TnI. However, the selective cleavage of TnI into short peptides using both chemical (e.g. cyanogen bromide) and enzymatic (e.g. trypsin) digestion has provided a tool for investigating biologically important regions in its primary sequence. There are two stretches of highly basic residues found in the primary sequence of TnI. Residues 102-135 contain 12 basic amino acids and residues 5-27 contain six basic amino acids (Wilkinson and Grand, 1975). Both of these segments are found to have significant role in regulating thin filament interactions (Syska *et al.*, 1976) and they will be investigated extensively in this thesis.

Troponin T (TnT) Skeletal TnT is composed of 259 amino acid residues and has a calculated molecular weight of 30,503. The protein carries an almost equal number of acidic and basic amino acid residues (61 Asp and Glu residues; 64 Arg, Lys and His residues) (Pearlstone and Smillie, 1976). Nevertheless, the pI value of TnT is 9.1, as determined by isoelectric focusing (Wilkinson, 1974). TnT is a highly polar molecule, with acidic side chains near its amino terminus (residues 1-39) and basic residues near its carboxyl terminal region (residues 221-259). Antibody experiments by Ohtsuki (1974, 1975 and 1979) demonstrated separate domains for the N-and C-terminal region of TnT along tropomyosin in thin filaments, suggesting an elongated rod shape for TnT at least 130 Å long. The carboxyl terminal region (about two-fifth) of TnT interacts with the central region of TM as well as TnI, TnC and actin (Ohtsuki, 1979; Pearlstone and Smillie, 1978 and 1985; Heeley and Smillie, 1988). The N-terminal region (about three-fifth) of TnT interacts with a long stretch of the carboxyl terminal region of TM and the head-to-tail overlap between TM molecule (Brisson *et al.*, 1986; White *et al.*, 1987). Isoforms of the TnT protein are found in vertebrate fast skeletal muscle. The sequence variations among the isoforms in a given species are limited to a region of approximately 30 amino acid residues near the N-terminus and another region of 14 amino acid residues near the C-terminus (Wilkinson *et al.*, 1984; Medford *et al.*, 1984; Breitbart *et al.*, 1985; Briggs *et al.*, 1989). The TnT isoforms are expressed in a tissue-specific and developmentally regulated manner (Medford *et al.*, 1984; Breitbart *et al.*, 1985). There is increasing evidence that the sequences of the N- and C-terminal variable regions of TnT may have significance contribution to the determination of the Ca^{2+}-sensitivity of muscle fibers (Greaser *et al.*, 1988; Nassar *et al.*, 1991; Pan and Potter, 1992; Schachat *et al.*, 1987; Tobacman and Lee, 1987). Phosphorylase kinase phosphorylates three sites in rabbit fast skeletal muscle TnT (Moir *et al.*, 1977); however a physiological role for TnT phosphorylation has not yet been demonstrated.

INTERACTIONS OF THIN FILAMENT REGULATORY PROTEINS

Tropomyosin (TM) and Troponin (Tn) interact with actin along the thin filament to regulate the actomyosin ATPase activity in the presence and absence of Ca^{2+}. The changes that occur in the protein interactions within the thin filament provide the mechanism of regulation of skeletal muscle contraction. Much of the current information on the detailed assembly of the thin filament proteins comes from studies on binary complexes. Major findings concerning the properties of these complexes are related to one another and they are summarized below.

I. Actin-TM-TnT Interactions

Each coiled-coil TM molecule spans seven polymerized actin monomers. The amino acid sequence of TM reveals 14 quasi-equivalent repeating regions of acidic and nonpolar residues throughout the length of each chain that could serve as actin-binding sites (Parry, 1974; Stewart and MacLachlan, 1975; Stone *et al.*, 1974). Therefore, in the TM dimer there would be 28 repeating acidic regions exposed on the surface of the molecule, arranged in 14 opposing pairs such that four sets of seven regions occur at 90° from each other (Stewart and MacLachlan, 1975). Each of these seven regions would allow contact with one actin monomer. These acidic regions may interact with acidic regions on actin through Mg^{2+} bridges, or with positive regions on actin via salt bridges. Hydrophobic interactions between nonpolar groups may also provide additional stability (Stewart and MacLachlan, 1975). Stewart and MacLahlan (1975) have proposed that one set of the seven binding sites attaches to actin in the relaxed muscle, and one set upon muscle activation, during which the TM molecule moves one-quarter turn to interact with actin from a different angle. This model is consistent with the steric blocking mechanism of regulation. Alternatively, Philips *et al.* (1980) have suggested that TM is continually changing connections with actin, producing a net effect of relaxation or contraction.

The head-to-tail overlap region of TM also appears to be important in the binding of actin (Mak *et al.*, 1981). The ability of TM to polymerize along actin greatly increases its binding affinity towards actin (Walsh and Wegner, 1980; Wegner, 1979), and TM that is unable to polymerize owing to the removal of its C-terminal 11 residues can no longer bind to actin (Mak and Smillie, 1983). The regions of TM and actin which are presumed to interact with each other are given in Table I-2.

TnT links the Tn molecule to TM. Regions of TnT that interact with TM have been determined by protein fragment studies (Jackson *et al.*, 1975; Pearlstone and Smillie, 1977; Ohtsuki, 1979; Pato *et al.*, 1981; Tanokura *et al.*, 1983). These regions have been identified as residues 1-151, and 197-259. Mak and Smilie (1983) found that the removal of C-terminal 11 residues from TM greatly reduced the affinity of TM for residues 1-151 of TnT. This suggests that the binding of this segment of TnT occurs at the C-terminus of TM, perhaps extending to the N-terminus of the overlapping TM molecule. Pearlstone and Smillie (1982) showed that Ca^{2+}, in the presence of TnC, does not affect the binding affinity of segment 1-151 of TnT for TM. Residues 197-259 of TnT are known to interact in proximity to TnI and TnC in a Ca^{2+}-sensitive manner in the presence of TnC (Pearlstone and Smillie, 1982, 1983). The TnI and TnC subunits are found near C190 of TM, so it can be assumed that segment 197-259 of TnT binds near C190. CD studies (Pearlstone and Smillie, 1978) demonstrate a high helical content in residues 71-151 of TnT, whereas 159-227 of TnT contain a heptapeptide repeat similar to that in the TM sequence (Parry, 1981). This may suggest an interaction between TM and TnT that may be coiled-coil in nature (Mak *et al.*, 1983). The interaction of TnT with the overlap sequence of TM may stabilize the overlap region and/or have a role in the transduction of the Ca^{2+}-binding signal for muscle activation (Flicker *et al.*, 1982; Phillips *et al.*, 1980). The regions of TnT and TM which are presumed to interact with each other are given in Table I-2.

Antibody (Ohtsuki, 1979) and EM experiments of rotary-shadowed molecules (Flicker *et al.*, 1982) have revealed the Tn complex as a globular mass near C190 of TM. This region is composed primarily of TnC, TnI, and a rod like tail (which is TnT) extending toward the C-terminus of TM. These studies suggest at least two regions of interaction between TnT and TM: one near C190, where TnC and TnI are found, and one along the C-terminal stretch of TM.

II. TnC-TnT-TnI Interactions

TnT attaches Tn to TM through interactions with TnC and TnI subunits. The detail molecular interactions between TnT and TnC in whole Tn is uncertain, however it is known that TnT is required for full Ca^{2+}-dependent, reversible regulation of ATPase activity, and must therefore interact either directly or indirectly with TnC.

Residues 159-227 at the C-terminus of TnT are implicated in the binding of TnC (Ohtsuki, 1979; Pearlstone and Smillie, 1978), and lysine residues within this region of TnT show reduced reactivity with acetic anhydride when TnC and TnT are mixed (Hitchcock *et al.*, 1981). Residues 1-70 at the N-terminus of TnT have been observed to bind to TnI (Pearlstone and Smillie, 1980), but a reinvestigation showed there was no interaction between these regions at lower, more physiological ionic strength (Pearlstone and Smillie, 1983). A second region of TnT (residues 159-259), however, has been shown to interact with TnI, according to gel chromatography and CD studies (Pearlstone and Smillie, 1978,1985). Investigations with peptides that overlap residues 159-259 of TnT (Pearlstone and Smillie, 1978) indicates that TnI binds at the N-terminal end of this sequence, since it binds to a peptide of residues 152-175. On the other hand, TnC binds at the C-terminal end of this region, since it binds to a peptide of residues 176-230 (Pearlstone and Smillie, 1978). This conclusion is consistent with the results of lysine reactivity studies (Hitchcock *et al.*, 1981) and phosphorylation studies (Moir *et al.*, 1977) of the TnT-TnI complex. The regions of TnT which are presumed to interact with TnC and TnI are given in Table I-2.

There may be several sites in the N-terminal region of TnC (residues 1-100) that are able to form Ca^{2+}-dependent interaction with TnT (Grabarek *et al.*, 1981; Weeks and Perry, 1978). Lysine reactivity in this region of TnC are reduced when TnC is complexed with TnI or TnT (Hitchcock, 1981), and TnT has been shown to crosslink to Cys-98 of rabbit skeletal TnC alone or in a Tn complex (Tao *et al.*, 1986a, 1986b). The regions of TnC that are presumed to interact with TnT are listed in Table I-2.

TnI Residues 57-106 contain a heptad hydrophobic repeat like that observed between residues 197-250 of TnT. This pattern of repeat is typical of a coiled-coil structure and this suggests there may be an interaction between these two regions on the TnI and TnT proteins (Pearlstone and Smillie, 1985). This hypothesis is supported by Chong and Hodges (1982b) who used an iodoacetamide labeling of cycteines to demonstrate that Cys-48 and Cys-64 of TnI are in proximity to residues 176-230 of TnT. Chong and Hodges (1982c) also used an iodoacetamide labeling of cysteines to show that Cys-48 and Cys-64 are inaccessible only in whole Tn and in a TnI/TnT complex, whereas Cys-133 of TnI, which is presumed not to associate with TnT, is always reactive. Hitchcock-DeGregori (1982) showed that several lysines in the region of residues 40-78 of TnI exhibited changed reactivity with acetic anhydride in the TnI/TnT complex. The reactivity of these lysines was sensitive to the binding of Ca^{2+} to TnC in whole Tn, which suggests that the interaction between TnI and TnT is Ca^{2+} sensitive. The region of TnI which are presumed to interact with TnT are given in Table I-2.

III. TnC-TnI-Actin Interactions

The Ca^{2+}-dependent interactions between TnC, TnI and actin are critical interactions in the activation of contraction in skeletal muscle. TnI inhibits the Mg^{2+}-

ATPase activity of actin-myosin (Hartshorne and Mueller, 1968; Schaub and Perry, 1969; Greaser and Gergely, 1971; Perry *et al.*, 1975) and binds both to actin and TM-actin (Potter and Gergely, 1974; Hitchcock, 1975). The inhibition of actomyosin ATPase activity is neutralized when Ca^{2+}-saturated TnC form a 1:1 complex with TnI (Perry *et al.*, 1975; Weeks and Perry, 1978; Chong *et al.*, 1983). Syska *et al.* (1976) initiated the investigation on the ability of TnI, CNBr and cysteine cleavage fragments of TnI to bind to TnC. Three TnI fragments, CN4 (residues 96-116), CN5 (residues 1-21) and CF2 (residues 1-47) were capable of binding to a TnC-Sepharose affinity column. CN4 fragment (residues 96-117) was also able to bind actin-TM and inhibited the acto-S1-TM ATPase activity (Syska *et at.*, 1976). Subsequent investigations have shown that TnI residues 104-115 comprises the minimum sequence that inhibits the actomyosin ATPase activity and binds to TnC causing the release of the ATPase inhibition (Talbot and Hodges, 1979, 1981a, 1981b; Katayama and Nozaki, 1982; Cachia *et al.*, 1983, 1986; Van Eyk and Hodges, 1987, 1988). Although it is very well documented that the TnI inhibitory region (residues 104-115) interacts with TnC (Talbot and Hodges, 1979, 1981b; Nozaki *et al.*, 1980; Katayama and Nozaki, 1982; Cachia *et al.*, 1983, 1986; Van Eyk and Hodges, 1987, 1988), the region on TnC that interacts with the inhibitory region of TnI is not clearly defined. Several physical experiments and chemical crosslinking studies have indicated that the TnI inhibitory peptide interacts with the C-domain of TnC (Weeks and Perry, 1978; Chong and Hodges, 1981, 1982b; Dalgarno *et al.*, 1982; Cachia *et al.*, 1983, 1986; Drabikowski *et al.*, 1985; Tao *et al.*, 1986; Leszyk *et al.*, 1987,1988; Lan *et al.*, 1989; Van Eyk *et al.*, 1991b; Swenson and Fredricksen, 1992). However, other crosslinking results demonstrated that residues from the regulatory Ca^{2+}- binding site II in the N-terminus of TnC (residues 46-78) form crosslinks with TnI inhibitory segment residues 92-167 (Leszyk *et al.*, 1990). Kobayashi *et al.* (1991) have shown that a mutant TnC containing a single Cys at residue 57 and modified with 4-maleimidobenzophenone can be crosslinked to the inhibitory region of TnI. The regions of TnI which are presumed to interact with TnC and actin are listed in Table I-2.

A number of studies on TnI/TnC interactions using proteolytic fragments of TnC have revealed three region of the TnC protein that are capable of interacting with TnI; the C-helix (residues 50-60), the E-helix (residues 89-100) and the G-helix (residues 126-136). The six lysines of TnC which demonstrate reduced reactivity with acetic anhydride (acetylation) upon TnC/TnI interaction are located within these three helices (Hitchcock, 1981). The interaction of TnI with the G-helix is Ca^{2+}-insensitive, while the interactions of TnI with the other two helices (C and E-helices) are Ca^{2+}-sensitive. NMR studies and other chemical accessibility studies (Hitchcock, 1981; Ingraham and Hodges, 1988) show that the residues along the D-helix become more exposed to solvent upon Ca^{2+} binding to TnC, potentially opening a new binding site for TnI or TnT. Recent site-directed mutagenesis studies on TnC and CaM showed that introduction of a disulfide bridge (it serves to block the key conformational change) into either the N or C-domain of the protein abolishes its interaction with the target protein. They suggested that the type of conformational change involving angular movement of the helical segment that takes place in TnC is also involved in signal transmission in other Ca^{2+}-dependent regulatory proteins (Gergely et al., 1993).

Lan *et al.* (1989) indicated that the preferred binding site for the TnI inhibitory peptide was the C-terminal domain of calmodulin (78-148) with an affinity comparable with that in intact calmodulin or TnC. However, there was binding to the N-terminal half of calmodulin (1-77) but the binding affinity was an order of magnitude less. Ip also inhibited the tryptic digest at the midpoint of the central helix linking the two domains of either TnC or calmodulin.

Swenson and Fredrickson (1992) have shown by intrinsic fluorescence that Ip containing a N-terminal Trp binds in a 1:1 stoichiometry to TnC and its fragments (N-

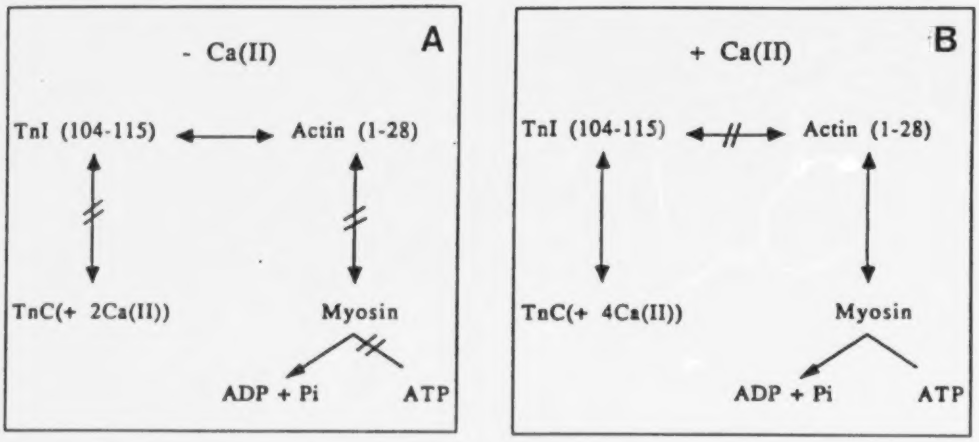
- Ca(II)
A
TnI (104-115)
Actin (1-28)
TnC(+ 2Ca(II))
Myosin
ADP + Pi
ATP
+ Ca(II)
B
TnI (104-115)
Actin (1-28)
TnC(+ 4Ca(II))
Myosin
ADP + Pi
ATP

Fig. I-7. **Schematic diagram showing the dominant protein interactions involved in the regulation of muscle contraction**. Panel A shows the dominant interactions when the Ca^{2+} specific sites I and II are unoccupied and muscle is relaxed. Panel B shows the dominant interactions when the Ca^{2+} specific sites I and II are occupied and muscle is contracted (Figure taken from Van Eyk *et al.*, 1991a).

terminal fragment, residues 1-97; C-terminal fragment, residues 98-159). Taken together the results of these authors and others (Van Eyk *et al.*, 1991b; Leszyk *et al.*, 1988, 1990; Heidorn and Trewhella, 1988; Cheung and Wang, 1989) have suggested that the binding of TnI inhibitory peptide may be more complex than initial interaction studies suggested and may form a single binding site between the N- and C-terminal domains of TnC.

The N-terminal region of actin (residues 1-28) can act as a chemical switch, flip-flopping between a binding site on the inhibitory region of TnI and a binding site on S1 (Van Eyk and Hodges, 1988; Van Eyk *et al.*, 1991b). The interaction that dominates depends on the relative binding affinities of the TnI inhibitory region (residues 104-115) and its interacting site on TnC and TM-actin, and the N-terminal region (residues 1-28) of actin and it's binding sites on TnI and S1. Since the interaction between actin/TnI and TnI/TnC depends on whether Ca^{2+} is bound to the low affinity sites (sites I and II) of TnC, the acto-S1-TM ATPase activity which resulted from the interaction between actin and S1 can then be related to Ca^{2+} binding to TnC. Van Eyk *et al.* (1991a) has proposed a schematic diagram (Fig. I-7) to describe the "flip-flop" action of the actin regulatory region (residues 1-28) and TnI inhibitory region (residues 104-115) involved in the regulation of muscle contraction and relaxation.

MODELS OF MUSCLE REGULATION

The present view for muscle contraction and relaxation process is shown in Fig. I-8. It consists of the sliding-filament theory, in which filaments of constant length past one another; the cross-bridge theory, which describes the propelling mechanism in which small transverse "cross-bridges" project from the thick filaments and interact cyclically with the thin filaments. Each interaction drives the thin filament past the thick filament (A. F. Huxley, 1957).

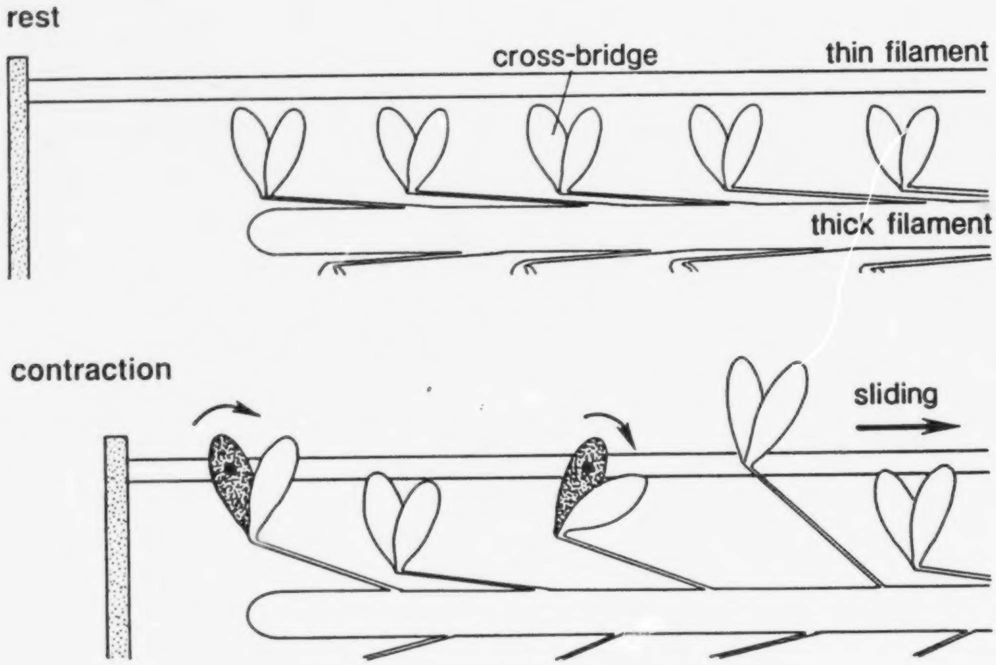
rest
cross-bridge
thin filament
thick filament
contraction
sliding

Fig. I-8. **Schematic description of the sliding-filament-cross-bridge theory**. The sarcomere shortens (bottom) as cross-bridges from thick filament attach to thin filament, rotate clockwise, and thereby drive thin filament rightward. Each cross-bridge may undergo many such strokes during a contraction.

I. Steric Blocking Model

The steric blocking model (Fig. I-9) is based on the three-dimensional reconstruction of negatively stained actin filaments decorated with S1 obtained from electron micrographs. There is an observed shift in the position of TM within the thin filament (Huxley, 1972; Haselgrove, 1972; Parry and Squire, 1973; Wakabyashi *et al.*, 1975). It was hypothesized that during muscle relaxation, TM was located in proximity to an actin-myosin site of interaction and physically blocks the interaction between actin and myosin. During muscle contraction, TM moves away from this 'blocking' site deeper into the groove of F-actin which promotes the actin-myosin interaction (power stroke). Further more, on the basis of the results of ATPase activity assay using various components of troponin, tropomyosin, actin and myosin S1; it was postulated that regulation of the TM position occurs through TnI. In the absence of Ca^{2+}, TnI is bound to actin-TM in such a way as to block actin-TM interaction with myosin, while in the presence of Ca^{2+}, the TnI interacts with TnC and allows interaction between actin-TM and S1 (Hitchcock *et al.*, 1973; McCubbin and Kay, 1980; Potter and Gergely, 1974). The steric blocking model implies that the movement of TM results not only the exposure of the myosin binding site on actin-TM, but also enhances the myosin ATPase activity.

II. Allosteric Blocking Model

The allosteric blocking model for regulation of muscle contraction is an attempt to link the biochemical process with the mechanical steps involved in the power stroke, Eisenberg *et al.*, (1980) proposed that actin and myosin cross bridges in muscle fiber can be in one of four different states (Fig. I-10 i, ii, iii and iv). In two of the states, the thin filament is not bound to myosin (i and ii) while in the other two states, the thin filament is bound (iii and iv) (Fig. I-10). The two bound states are assumed to have very different mechanical properties from one another. The initial cross bridge attachment between the

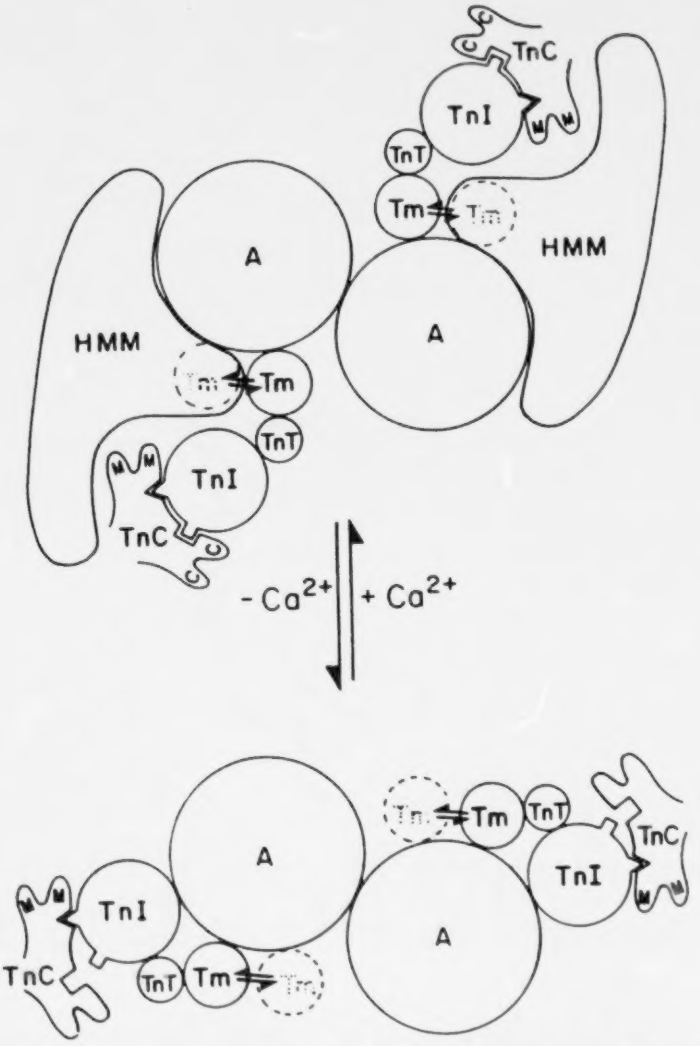
TnC
TnI
TnT
Tm
Tm
HMM
A
A
HMM
Tm
Tm
TnT
TnI
TnC
- Ca^{2+}
+ Ca^{2+}
A
Tm
Tm
TnT
TnC
TnI
A
TnI
TnC
TnT
Tm
Tm

Fig. I-9 **Cross section of skeletal muscle thin and thick filaments illustrating possible change of interactions upon the binding of Ca^{2+} to TnC**. A, actin; HMM, heavy meromyosin; TnC, troponin C; TnI, troponin I; TnT, troponin T; Tm, tropomyosin (Figure taken from Potter and Johnson, 1982).

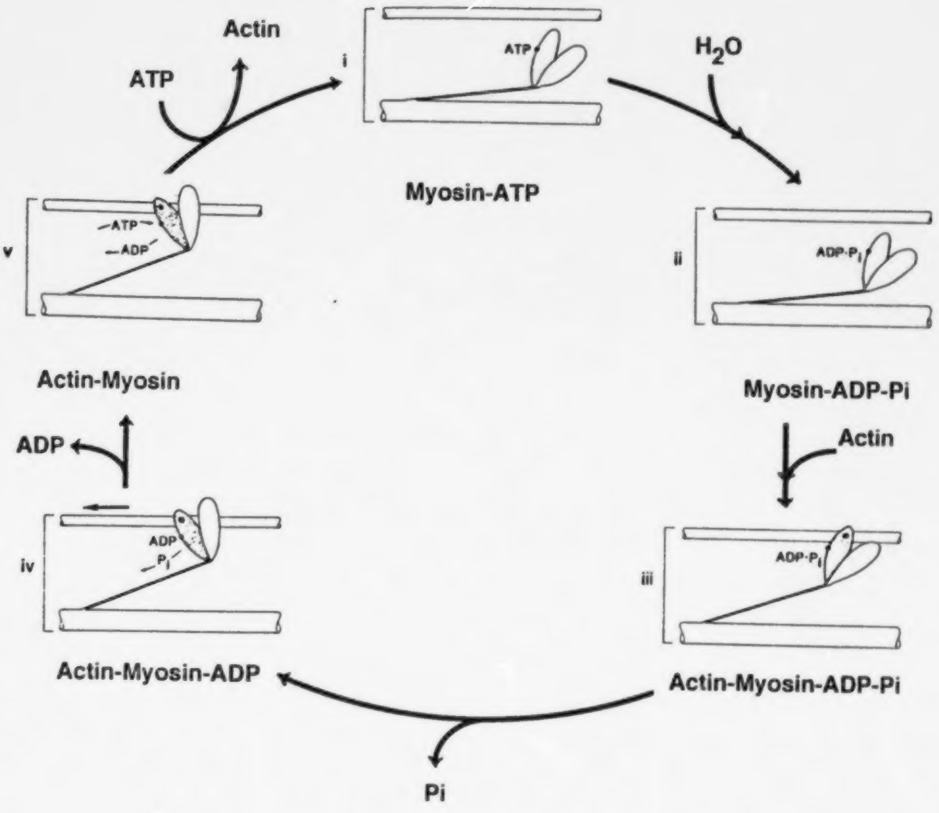
Actin
ATP
i
ATP
H$_2$O
Myosin-ATP
v
ATP
ADP
ii
ADP·P$_i$
Actin-Myosin
Myosin-ADP-Pi
ADP
Actin
iv
ADP
P$_i$
iii
ADP·P$_i$
Actin-Myosin-ADP
Actin-Myosin-ADP-Pi
Pi

Fig. I-10. **Simplistic scheme for the coupling of ATP hydrolysis cycle to kinetic steps involved during muscle contraction.** Pi is inorganic phosphate (see text for detail).

actin and myosin is weak (Acto-myosin-ADP-Pi). Upon phosphate release, there is a transition to a strongly bound state (Acto-myosin-ADP). The transition from the weak binding state to the strong binding state (Fig. I-10, iii to iv) of actin-myosin corresponds to a 90° to 45° movement of the myosin head (power stroke). Removal of the ADP yields the rigor complex (v) (Actin-myosin complex in a ATP-free state) which is dissociated upon binding of ATP to myosin head to yield Myosin-ATP (i) and thus, completing the contraction cycle.

An expansion of the allosteric model proposes that TM-Tn is capable of blocking the movement of the myosin head from 90° to 45° and thus, promoting muscle relaxation (Chalovich *et al.*, 1983). However, TM may not necessarily physically block actin-myosin interaction as predicted by the steric blocking model but rather prevent the release of Pi from the nucleotide active site (Fig. I-10, iii to iv), possibly through conformational change in actin and/or myosin (Lehrer and Morris, 1982). Nevertheless, Hill (1983) proposes that TM occupies several positions on the thin filament that are under the control of S1 and Tn. The position to which TM is shifted (which is regulated by Tn) when S1 binds depends on which nucleotide is bound to S1 (since the particular nucleotide may affect the angle at which S1 attaches to actin) and on the number of moles of Ca^{2+} bound to TnC that affect the conformation of the thin filament (Hill, 1983; Hill *et al.*, 1984). This suggests that there is a relationship between the number of cross bridge attachments or strength of each crossbridge (actin-myosin interaction) and Ca^{2+} binding to TnC of the Tn complex on the thin filament. There is some evidence from previous work to support this idea. Bremel and Weber (1972) demonstrated that the presence of rigor cross bridges (strong interaction between myosin and actin in the absence of nucleotide) increases the Ca^{2+} affinity of Tn and suggested that cross bridge attachment affects the conformation of TnC. The interaction of TM-Tn with actin lowered the affinity of the regulatory sites of TnC (Zot *et al.*, 1983; Zot and Potter, 1987), suggesting a relationship between actin and TnC conformation.

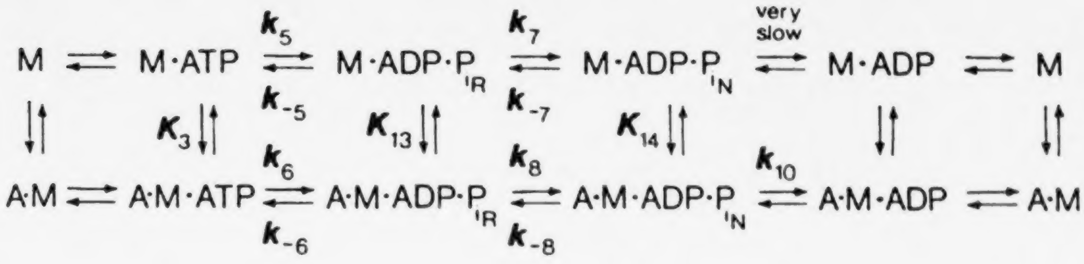
M
M·ATP
M·ADP·P_iR
M·ADP·P_iN
M·ADP
M
A·M
A·M·ATP
A·M·ADP·P_iR
A·M·ADP·P_iN
A·M·ADP
A·M
k_5
k_-5
k_7
k_-7
very slow
K_3
K_13
K_14
k_6
k_-6
k_8
k_-8
k_10

Fig. I-11. **Currently popular "modified-refractory-state model" relating cross-bridge cycling to ATP splitting**. The labels K and k denote equilibrium constants and rate constants, respectively. M = myosin; A = actin; Pi = inorganic phosphate. After Stein *et al.* (1979).

Recently, by incorporating a fluorescence probe (Danz) labeled TnC into TnC-depleted muscle fibers, it was shown that there were different TnC conformations and Ca^{2+} affinities depending on whether the myofibril was in a weak or strong cross bridge state (Zot and Potter, 1989). Taken together, these results suggest that conformational changes due to cross bridge formation between actin and myosin influences the structural changes that occur when Ca^{2+} binds to TnC and vise versa. The difficulties relating ATP hydrolysis to cross-bridge cycling go beyond those models mentioned above. In essence, the scheme of Fig. I-10 has proved too simple to account for a number of observations on actomyosin kinetics. The models have been repeatedly modified or amended and a current version of the scheme, the "modified-refractory-state model," is shown in Fig. I-11 This model contains ten cross-bridge states and other models contain more.

The nature of the conformational differences in the S1 head due to the presence of a particular nucleotide, the conformational change induced by actin binding (hence the position of TM-Tn on the actin filament), and changes induced by Ca^{2+} binding to TnC are unclear. In order to clarify our understanding of these conformational changes in the thick and thin filaments, the exact sites of interaction between the various muscle proteins must be identified both in the presence and absence of Ca^{2+}.

PURPOSE OF THIS STUDY

This thesis has focused on studies of the TnI/TnC interaction and has allowed us to determine the exact binding site on TnC for the TnI inhibitory peptide (Ip). A photoactivatable radioactive TnI inhibitory peptide, residues 104-115 (BBIp) was synthesized by solid-phase methodology and crosslinked to TnC in the presence and absence of calcium. Characterization of the crosslinked TnC/BBIp complex is performed (Chapter III). Based on these (and previous) crosslinking results together with the x-ray crystallographic structure of TnC (Herzberg and James, 1985, 1988) and the knowledge of the structure of Ip when bound to skeletal TnC (Campbell and Sykes, 1989 and 1991; Campbell *et al.*, 1991), we generated a three-dimensional model of the TnC C-domain/Ip complex.

Although the inhibitory site (residues 104-115) of TnI is of major importance, it is not the only Ca^{2+} -sensitive TnI/TnC binding site (Syska *et al.*, 1976; Weeks and Perry, 1978 and Grabarek *et al.*, 1981). Our other investigations have focused on the N-terminal region of TnI. Synthetic peptides corresponding to the fragments of the N-terminus of TnI were synthesized and used to investigate the biologically important interaction between TnC and the N-terminal region of TnI. Characterization of the binding of the TnI N-terminal peptide to TnC was performed using an ATPase assay, by circular dichroism, HPLC, gel electrophoresis, crosslinking and fluorescence studies (Chapter IV, V and VI).

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CHAPTER II

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and solvents were reagent grade. Diisopropylethylamine (DIEA), trifluoroacetic acid and dichloromethane (DCM) were distilled prior to use. Picric acid was dissolved in dichloromethane and dried over magnesium sulfate. Acetonitrile and water (both are HPLC grade) for running High Performance Liquid Chromatography were obtained from Fisher. Distilled water was purified by passage through a Milli-Q water purification system. Boc-amino acids were purchased from Bachem Fine Chemicals (Torrance, CA), Protein Research Foundation (Peptide Institute, Osaka, Japan), and Institut Armand Frappier (Quebec, Canada). The copoly (styrene-1% divinylbenzene) benzhydrylamine hydrochloride resin was purchased from U.C.B. Bioproducts, Belgium and Institut Armand Frappier Laval (Quebec, Canada).

PEPTIDE SYNTHESIS AND PURIFICATION

All peptides were prepared using the standard procedures for solid-phase peptide synthesis (Erickson and Merrifield, 1976) on an Applied Biosystems 430A solid phase peptide synthesizer (Foster City, CA). Peptides were synthesized following the general procedure for solid-phase synthesis described by Parker and Hodges (1985) and Hodges *et al.* (1988). All amino acids used were protected at the α-amino position with the t-butyloxycarbonyl (Boc-) group (Bachem, Philadelphia, PA). The following side-chain protecting groups were used: Arg(Tosyl), Asp(OBzl), Glu(OBzl), His(DNP), Lys(2-ClZ) and Thr(Bzl). All amino acids were double coupled using dicyclohexylcarbodiimide generated symmetric anhydrides in dimethylformamide (DMF) for the first coupling and dichloromethane (DCM) in the second coupling to co-poly (styrene, 1% divinylbenzene) benzhydrylamine-hydrochloride resin at a substitution of 0.9 mmol of NH_2/g of resin

(Bachem, Philadelphia, PA). Any incomplete couplings (99.2% yield or less as determined by a quantitative ninhydrin test) were coupled a third time manually using Boc-amino acids : [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] : 1-hydroxybenzotriazole : 4-methylmorpholine : active sites on resin 2:2:2:3:1 in N-methylpyrrolidone. The following steps were performed in the reaction vessel for each double coupling : 1) deprotection of the Boc-group with 33% trifluoroacetic acid (TFA) in DCM for 80 secs. 2) 50% TFA in DCM for 18 min. 3) three DCM washes, 4) 10% diisopropylethylamine (DIEA) in DMF 1 min. 5) 10% DIEA in DMF 1 min. 6) five DMF washes, 7) first coupling 30 min. 8) three DMF washes, 9) 10% DIEA in DMF for 45 sec. 10) one DMF wash, 11) three DCM washes, 12) second coupling period 30 min., 13) one DMF wash, 14) five DCM washes. If required, final acetylation was performed on the instrument using acetic anhydride : DIEA : mmol of peptide resin 50:20:1 for 10 min., then 100:20:1 for 5 min. in DCM. The completed peptides were cleaved from the resins with anhydrous hydrogen fluoride (20 ml/g of peptide resin) in the presence of 10% anisole and 1% ethanedithiol for 1 hour at -4° C using type 1B HF-Reaction Apparatus (Peninsula Laboratories Inc., Belmont, CA). The peptide-resin was then washed three times with diethylether (25 ml each). Then, the cleaved peptide was extracted from the resin with neat acetic acid (three times 25 ml each) and then lyophilized. The peptides were dissolved in 25% acetonitrile 75% water (80 mg/5 ml) and sonicated for 10 min. Neat acetic acid was added dropwise while sonicating until the sample cleared (5-10%). The samples were then spun down at 14,000 rpm for 2 minutes using an Eppendorf centrifuge 5414C (Fisher Scientific). The supernatant was then syringe filtered using a Millex-6V 0.22 μM filter unit (Millipore, Bedford, MA). This solution was then purified using reversed-phase chromatography using an Applied Biosystems 400 solvent delivery system and a 783A programmable absorbance detector connected to a Synchropak RP-4 (250 x 21.2 mm I.D.) reversed-phase column (Synchrom Inc., Lafayette, IN) operated at a flow rate of 2 ml/min. with a linear AB gradient where solvent A was 0.05% aqueous TFA and solvent B was

0.05% TFA in acetonitrile. The gradient rates varied between 0.1%-0.5% B/min. depending on the sample load used (Mant *et al.*, 1987a; Burke *et al.*, 1991 and Hodges *et al.*, 1991). The sample loads varied between 20 and 50 mg per run. The fractions were then analyzed using a HP1090 Liquid Chromatography (Hewlett Packard, Avondale, PA) using the above solvent system at 2% B/min. starting in 100% solvent A on a Zorbax R_X-C8 2.1 mm x 15 cm (Rockland Technologies, Giberstville, PA). The homogeneity of the purified peptide was determined by reversed-phase chromatography, amino acid analysis using a Beckman 6300 High Performance Analyzer (Allendale, NJ) and mass spectrometry using a BioIon 20 Plasma Desorption Time of Flight Mass Spectrometer (Uppsala, Sweden).

PROTEIN PURIFICATION

Myosin subfragment 1 (S1) was prepared by the method of Weeds and Taylor (1975), as modified by Talbot and Hodges (1981). The critical steps in S1 preparation are the experimental condition during chymotryptic digestion of myosin. After extraction and washing of myosin from the skeletal muscle mince, it was precipitated by dialysis against a buffer consisting of 0.12 M NaCl, 0.013 M Na_2PO_4, 0.007 M NaH_2PO_4, 1 mM EDTA, pH 7.0. This was brought to room temperature with gentle stirring and the myosin concentration diluted until it was 15 mg/ml. Chymotrypsin, dissolved in less than 10 ml of the dialysis buffer, was added to the myosin solution to a concentration of 0.05 mg/ml (1:200, w/w). The digestion was allowed to continue for 20 min. and was terminated with 0.1M PMSF in minimal isopropanol for 15 minutes to allow complete inhibition to occur. The solution of S1 and undigested myosin was dialyzed against 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM NaN_3, pH 7.0. The precipitate was removed by centrifugation at 45,000 x g for 30 min. The supernatant was applied to a DEAE-52 cellulose column (2.8 x 45 cm) which had been equilibrated with the same buffer used in the last dialysis. The S1 was eluted with a linear gradient made of 500 ml of the above buffer and 500 ml of the same buffer which had been 0.2 M in KCl. The column was run at 40 ml/hr and fractions were collected every 15 min. A typical elution profile for the isolation of the S1 is shown in Fig. II-1. The two peaks (S1-A1 and S1-A2) found by reading fractions at 280 nm were collected and concentrated (if necessary) on a Amicon ultra filtration device equipped with a PM-10 filter. The S1 was dialyzed against 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM NaN_3 buffer, pH 7.0. For storage, a solution of S1 and sucrose in a 1:5 ratio (w/w) could be frozen rapidly in liquid N_2 then stored at -40 °C for up to a year. Prior to use, the S1-sucrose solution was extensively dialyzed against ATPase buffer containing 1 mM DTT. The K^+-EDTA ATPase activity for the various S1 (A_1,A_2) preparations varied between 500 and 600 nmole of Pi released/min./mg of S1.

Rabbit skeletal Tn, TnI and TnC were prepared by the procedures of Chong and Hodges (1982a, 1982b). To prepare Ca^{2+}-free TnC, TnC was denatured in 6 M guanidine hydrochloride in the presence of excess DTT and EDTA, then dialyzed against the appropriate buffer. 1 mM DTT was added to the final dialysis. Rabbit cardiac α-TM was extracted and purified as described by Pato *et al.* (1981). G-actin was purified from rabbit skeletal muscle acetone powder as described previously (Spudich and Watts, 1971). The purity of all proteins was checked by sodium dodecyl sulfate urea-polyacrylamide gel electrophoresis (12% crosslinked) and reversed-phase HPLC. The concentrations of all proteins and synthetic peptides were determined by amino acid analyses except S1 which were determined by absorbance (Yagi *et al.*, 1967).

POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

With the exception of SDS gel electrophoresis, all preincubated mixtures of proteins and peptides were obtained by dissolving the corresponding components in a buffer consisting of 20 mM Tris-HCl, 50 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$ or 3 mM $CaCl_2$ at pH 7.5-8.0 under nitrogen atmosphere, and were allowed to equilibrate (while stirring) at 4 °C for at least 3 hours before loading onto the gel.

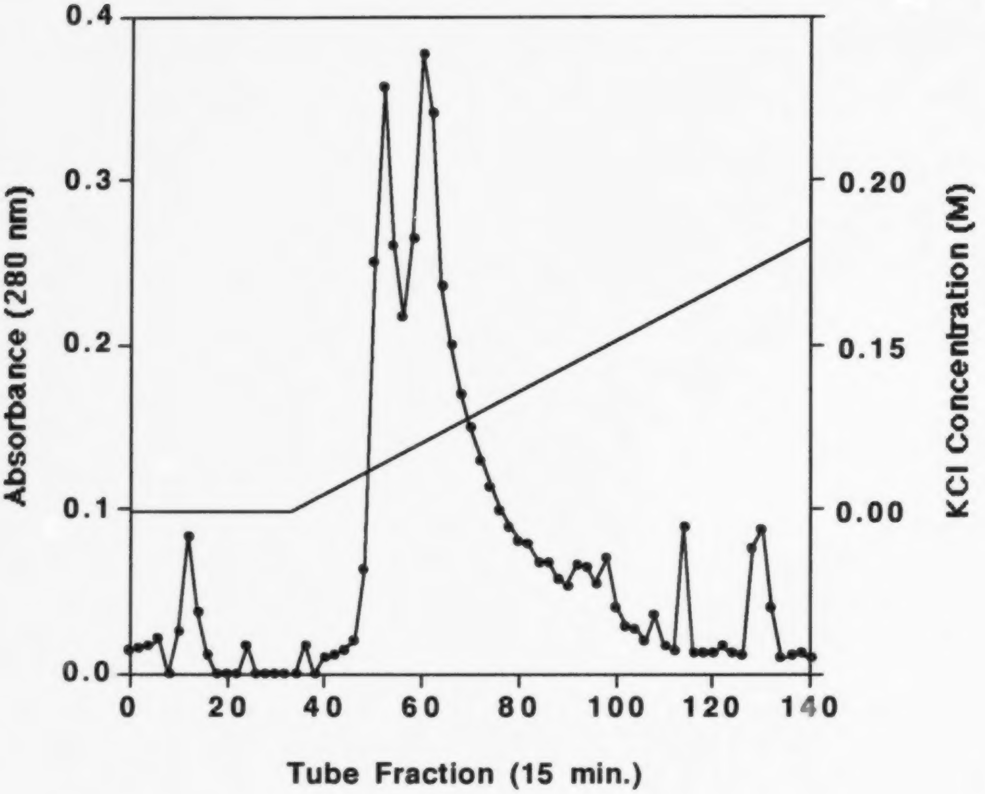

Fig. II-1 **DEAE-52 cellulose purification of rabbit skeletal S1** KCl Concentration is determined by conductivity (see Materials and Methods section for experimental conditions.

SODIUMDODECYL SULFATE (SDS) PAGE. All SDS gel electrophoresis were done with a 12% acrylamide 6M urea gel using the procedure of Weber and Osborne (1969). The gels were run on Protean dual slab cell apparatus (Biorad, Richmond, CA) giving a gel size of 14 x 16 cm. Samples were dissolved in 8 M urea, 0.1 M H_3PO_4-Tris, 1% SDS and 1% DTT, pH 6.8. A solution of bromophenol blue dye was used for tracking. After electrophoresis, gel were stained with a 0.25% solution of Coomasie brilliant blue dye in methanol, acetic acid and water (1:1:8, v/v/v).

NATIVE ALKALINE PAGE. All native alkaline polyacrylamide gel electrophoresis were performed by the modified method of Head and Perry (1974) on gel slabs made from 8% polyacrylamide gel, containing either 3 mM $CaCl_2$ (in the presence of calcium) or 5 mM $MgCl_2$ with 0.1 mM EGTA (in the absence of calcium) in 20 mM Tris/124 mM glycine buffer at pH 8.6.

UREA ALKALINE PAGE. All urea alkaline polyacrylamide gel electrophoresis were performed by the modified method of Head and Perry (1974) on gel slabs made from 8% polyacrylamide gel, containing either 3 mM $CaCl_2$ (in the presence of calcium) or 5 mM $MgCl_2$ with 0.1 mM EGTA (in the absence of calcium) and 6M urea in 20 mM Tris/124 mM glycine buffer at pH 8.6.

ATPase ASSAYS

The purified proteins used in the ATPase assays were prepared as follows: tropomyosin and TnC (~3 mg/ml) were dissolved in the Mg^{2+}-ATPase assay buffer (minus ATP, plus 2 mM DTT). Due to solubility problems, TnI (~3 mg/ml) was dialyzed in 8 M urea, 1 M KCl, 2 mM DTT, 10 mM Tris buffer, pH 7.8. The TnI sample was next dialyzed against 1 M KCl, 2 mM DTT 10 mM Tris buffer, pH 7.8, and then finally against 0.5 M KCl, 2 mM DTT, 10 mM Tris buffer, pH 7.8. The dialysis of TnI was carried out under a nitrogen atmosphere to prevent oxidation of cysteine residues (Horwitz *et al.*, 1979). S1 and G-actin (G-actin will be polymerized to F-actin in 30 mM KCl Mg^{2+} ATPase buffer) were dialyzed against the ATPase buffer (minus ATP, under a nitrogen atmosphere). The TM to actin ratio used in these experiments was 1:7. The actin and S1 ratios varied with each experiment.

ATPase assays were performed using an automatic pH-stat apparatus consisting of a Brinkman Metrohm 614 Impulsomat, 655 Dosimat, 635 Dosigraph and 635 pH meter with a 1 ml syringe (Côté and Smillie, 1981; Van Eyk and Hodges, 1991). Assay samples, 2 ml in volume, were placed in glass vials and stirred continuously at 25 °C. The acto-S1-TM ATPase activities were measured in a buffer consisting of 5 mM Tris, 30 mM KCl, 0.1 mM EGTA, 5 mM $MgCl_2$ and 2.5 mM disodium ATP, pH 7.8. For experiments requiring the presence of calcium the same buffer was used except the 0.1 mM EGTA was replaced with 3 mM $CaCl_2$. The titrant was 5-10 mM standardized KOH. A single assay vial of acto-S1-TM was titrated with the protein(s) or peptide(s) in the same buffer, and the effect of accumulated protein or peptide on the ATPase activity was determined after each consecutive addition of the protein or peptide. To ensure the ATPase assay was functioning correctly, the experiment was performed with each new S1 and F-actin preparation.

CENTRIFUGATION STUDY

Radioactive acetic anhydride, [1-^{14}C] was used for the manual acetylation of the N-terminal amino group of Ip (TnI 104-115) with a mixture of acetic anhydride/toluene/pyridine (1:3:3 v/v), a radioactive label was incorporated to produce a specific activity of 2723 cpm/nmole of Ip (Chapter IV).

To determine whether the TnI peptide (TnI1-40, Rp or TnI104-115, Ip) could bind to the Actin-TM complex, a mixture of actin (10 nmoles), tropomyosin (1.4 nmoles) and TnI peptide (2 nmoles) ± TnC (4 nmoles) were incubated in 200 μl of Mg^{2+}-ATPase Buffer (± Ca^{2+}) at room temperature for 15 minutes and then centrifuged for 1 hr. at

175,000 x g at 25 °C in a A-110/30 rotor of a Beckman Airfuge. Aliquots (100 µl) of the supernatant were taken either for radioactive measurement (in the case of Ip-^{14}C) or for reversed-phase chromatography (in the case of TnI1-40, Rp) to quantify the peptide remaining in the supernatant, thus, determining the amount of peptide bound in the Actin-TM pellet (see Chapter IV for experimental results).

SIZE EXCLUSION CHROMATOGRAPHY

Mixtures of skeletal TnC (or recombinant TnC mutants) and TnI N-terminal peptide (TnI1-40, Rp or TnI1-30) were dissolved in a buffer consisting of 20 mM Tris-HCl, 50 mM KCl, 3mM $CaCl_2$ and 0.1 mM DDT at pH 6.8 were pre-incubated at 4 °C under nitrogen atmosphere for 1 hour. The TnC/peptide (or TnC domain/peptide) mixtures were loaded onto a high performance size-exclusion column, Altex TSK G2000 SW (7.5 mm I.D. x 30 cm) (Beckman Inc., Berkeley, CA) in a buffer consisting of 20 mM Tris, 100 mM KCl, in the presence of 3 mM $CaCl_2$ and 0.1 mM DTT, pH 6.8, at a flow rate of 0.4 ml/min. at room temperature. The HPLC system used for the size-exclusion chromatography (SEC) was a Vista series 5000 liquid chromatograph (Varian, Walnut Creek, CA), HP 1040A detector, 8513 computer, HP9121 disc drive, HP25A inkjet printer and HP7470A plotter (Hewlett Packard, Palo Alto, CA). The salt was present in the buffer to eliminate non-specific ionic interactions between the peptide or protein and the support, and hence maintain ideal size-exclusion behavior (Mant *et al.*, 1987b). Peaks of interest were collected and analyzed by microbore reversed-phase chromatography on a microbore column (Aquapore RP-300 (C_8), 100 x 1.0 mm I.D. 300 Å pore size and 7 µm particle size) (Brownlee Labs., CA, U.S.A.). The peptides and proteins were eluted from the microbore column by employing a linear A-B gradient (2% B/min.) where eluent A is 0.05% aqueous TFA and eluent B is 0.05% TFA in acetonitrile (pH 2.0), flow-rate, 0.2 ml/min. at room temperature. The reversed-phase chromatography on the microbore column was carried out on an HP1090 fully automated liquid chromatography containing a

HP8513 computer, HP9133 disc drive, 9000-300 hard disc and HP 7470A plotter. To calculate the peptide/protein ratio in the complex, the peak areas of peptide and TnC obtained on separation of the complex by the microbore reversed-phase chromatography were compared to the peak areas of standard solutions of peptide and TnC. The quantity of peptide or protein in the standard solutions was determined by amino acid analysis and used to calculate an instrument and column dependent extinction coefficient (mAu/nmole) for both TnC and peptide (Chapter IV and VI).

CROSSLINKING STUDIES

In Chapter III, TnC (30 μM) and BBIp (100 μM) at a molar ratio of 1:3.3 were pre-incubated in a buffer consisting of 20 mM Tris-HCl, 0.1 mM EGTA, 5 mM $MgCl_2$ (Mg-buffer) or 3 mM $CaCl_2$ (Ca-buffer) at pH 7.0 under a nitrogen atmosphere, and were allowed to equilibrate (while stirring) at 4 °C for one hour. Whereas in Chapter V, TnC (30 μM) and BBRp (60 μM) at a molar ratio of 1:2 were pre-incubated under three conditions : 1) 20 mM Tris-HCl, 3 mM EDTA (metal ion free), 2) 20 mM Tris-HCl, 0.1 mM EGTA and 5 mM $MgCl_2$ (Mg^{2+}-buffer) and 3) 20 mM Tris-HCl, and 3 mM $CaCl_2$ (Ca-buffer) at pH 7.0 under nitrogen atmosphere. The mixtures were allowed to equilibrate (while stirring) at 4 °C for one hour. All equilibrated mixtures were subjected to photolysis by exposing the mixture to ultraviolet irradiation for one hour at 4 °C in an RPR 208 preparative reactor (Rayonet, The Southern New England Ultraviolet Co., Middletown, CN) equipped with RPR 350 nm lamps (Fig. II-2). Completeness of the photochemical crosslinking reaction was monitored by SDS gel electrophoresis. The photolyzed protein/BB-peptide mixtures obtained were freeze-dried and the crosslinked complex was isolated by reversed-phase HPLC (see Chapter III for figure).

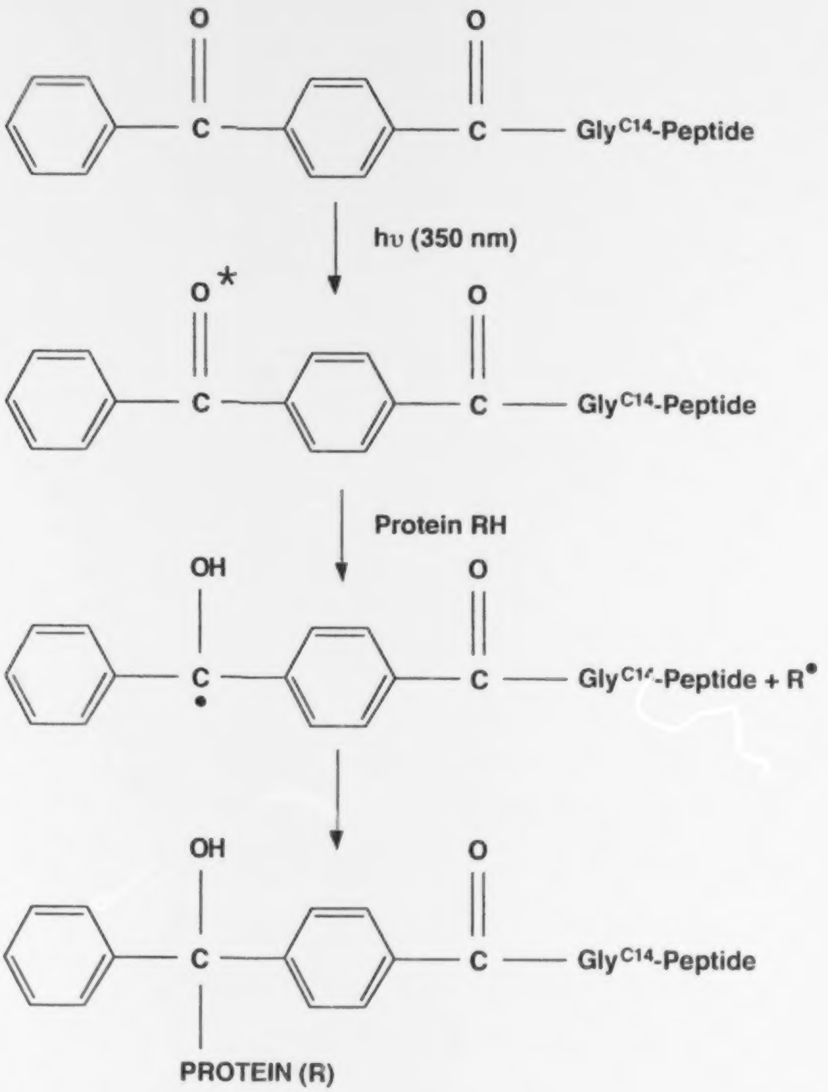
O
O
C
C
GlyC14-Peptide
hυ (350 nm)
O*
O
C
C
GlyC14-Peptide
Protein RH
OH
O
C
C
GlyC14-Peptide + R•
OH
O
C
C
GlyC14-Peptide
PROTEIN (R)

Fig. II-2 **Schematic diagram of a photoaffinity labile peptide and its target protein**. A radioactive ^{14}C-labeled Gly was incorporated at the N-terminus of the TnI peptide. The photoaffinity label (Benzoylbenzoyl, BB) is stable in the peptide-protein complex until activated by exposure to 350 nm light source (Figure taken from Bayley, H. 1983).

TRYPTIC DIGESTION OF TNC/BBIP COMPLEX

Complete tryptic digestion of the isolated TnC/BBIp complex (Chapter III) was performed using a 1:20 molar ratio of trypsin-TPCK (5 μM) to protein (100 μM) incubated at 37 °C in 50 mM NH_4HCO_3 and 5 mM EDTA (amount of the photolyzed complex varied between 20 and 40 mg per run). After 16 hours of tryptic digestion, equal volume of 0.05% aqueous TFA was added to the mixture to stop the digestion and the reaction mixtures were then lyophilized. The corresponding lyophilized digest was loaded onto an analytical reversed-phase column (Aquapore RP-300 (C_8), 220 x 4.6 mm I.D., 7 μm, 300 Å; Brownlee Labs., Santa Clara, CA, U.S.A.) operated with a linear AB gradient at a flow rate of 2 ml/min. where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile. The gradient rate was 0.5 % B/min.. The photochemical crosslinked fragment was detected by radioactive counting of the one minute fractions that were collected from the reversed-phase run of the digested mixture. The major radioactive fraction was collected and lyophilized for further characterization.

CYANOGEN BROMIDE CLEAVAGE

CNBr cleavage of the radioactive tryptic fragment was carried out using a 200-fold molar excess of cyanogen bromide over methionine of the fragment (2000 μM CNBr to 10 μM radioactive tryptic fragment) in 1 ml of 70% formic acid at 25 °C. After 24 hours the reaction mixture was diluted 10-fold with water and was lyophilized to remove excess reagents (Chapter III).

AMINO ACID SEQUENCING AND MASS SPECTROMETRY

Amino acid sequence analysis was performed with an Applied Biosystems model 473A Protein Sequencer. Sample loadings varied between 200 and 400 pmoles (determined by radioactivity) per run. Mass spectra were recorded on a BIOION 20 plasma desorption mass spectrometer (Bio-Ion Nordic AB, Applied Biosystems Inc., Foster City, CA).

Samples (100-250 pmoles) were applied to a nitrocellulose coated mylar disc in 50:50 mixture of 0.05% aqueous TFA and ethanol, respectively. The sample solutions were allowed to absorb to the discs for 20 minutes and were spun dried. The spectra were collected for 3 hours at 16 kV and were calibrated using H^+ and NO^+ as internal standards (Chapter III).

COMPUTER MODELING

In chapter III, the simulations of the TnC/Ip complex structure were carried out on a Silicon Graphics Crimson Elan using the Insight II and Discover program (Biosym Technologies Inc., San Diego, CA). The X-ray crystal structure of turkey TnC (Herzberg and James, 1985, 1988) and the NMR-derived structure of TnI peptide bound to TnC (Campbell and Sykes, 1991) served as starting structures. The peptide was manually placed in the hydrophobic pocket of the C-terminal domain of TnC with an approximate distance of 5 Å between the N-terminal residue of Ip and Met 155 of TnC. A rigid body docking procedure similar to the Monte Carlo simulations described by Goodsell and Olson (1990) was employed. The interaction energy was defined as the sum of intermolecular Van Der Waals-energy and Coulomb-energy, the latter calculated in vacuo (e= 1). The MC-structure with the lowest intermolecular energy was subsequently energy minimized using the CVFF-forcefield. This procedure, the alternating application of Monte Carlo docking and energy minimization, was repeated until no further decrease in intermolecular energy was observed (20 times). During the first 15 cycles, only side chain atoms were allowed to move in the energy minimization, while the coordinates of backbone atoms of protein and peptide were fixed. During cycle 15 to 20 all atom positions were minimized.

Solvent accessible areas were determined using the program ANAREA (Richmond, 1984). The final modeled structure was used for the determination of the surface area of the complex as well as for TnC and Ip after deleting the other component of the complex.

Fractional areas were determined relative to the surface area in an extended chain (Shrake and Rupley, 1973).

FLUORESCENCE SPECTROSCOPY

In Chapter V, the intrinsic Trp fluorescence was determined by using a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with the DCSU-2 corrected spectra accessory which allows for automatic subtraction of fluorescence due to solvent. The instrument was operated in the ratio mode, and thermostated cells were maintained at 20 °C. Detection of fluorescence was effected at 90° to the excitation beam. The emission and excitation wavelengths were 301 and 282 nm, respectively (Lakowicz, 1983). Aliquots of TnI1-30 peptide were added to the initial 1000 μl solution of TnC mutant. The concentrations of TnC mutant are in the range of 4 to 5 μM. Emission spectra were determined from 280 nm to 400 nm. Solutions of proteins were obtained by initially denaturing TnC mutants in 6M guanidine hydrochloride in the presence of excess EDTA and DTT (Golosinska *et al.*, 1991; Pearlstone *et al.*, 1992), and subsequent dialysis of the protein against a buffer consisting of 50 mM Tris-HCl, 50 mM KCl and 2 mM $CaCl_2$ at pH 7.1 under nitrogen atmosphere (1 mM DTT was added to the final dialysis buffer). The purified TnI1-30 peptide was dissolved in the same buffer. Prior to spectral analysis, TnC and TnI1-30 samples were centrifuged in pre-rinsed Spin-X tubes (Costar) equipped with 0.22-μm nylon filter. The protein and peptide concentrations were determined by amino acid analysis using a Beckman 6300 acid analyzer.

CIRCULAR DICHROISM SPECTROSCOPY

The circular dichroism (CD) measurements were conducted on a JASCO J-720 spectropolarimeter (Jasco Inc., Easton, MD) interfaced to an Epson Equity 386/25 and conducted by Jasco software. The thermostated cell holder was maintained at 25 °C with a Lauda RMS circulating water bath (Lauda. Westbury NY). The instrument was routinely calibrated with ammonium d-(+)-10 camphor sufonate at 290.5 nm, and with d-(-)-pantoyllactone at 219 nm. Each sample was scanned ten times and noise reduction applied to remove the high frequency before calculating molar ellipicities. The voltage to photomutiplier was kept below 250 nm were 0.02 and 0.05 (calibrated for pathlength). The concentrations of the skeletal TnC varied between 22 and 30 M in the absence and presence of 1 molar equiv. of TnI1-30 peptide. The experimental data were analyzed by using a computer software program designed to determine biphasic binding curves (program kindly provided by Dr. B.D.Sykes, University of Alberta). the fitting program analyzed data in the form of the following equation:

$$Z = f_1 \frac{[Ca^{2+}]^{n1}}{[Ca^{2+}]^{n1} + Kd_1^{n1}} + f_2 \frac{[Ca^{2+}]^{n2}}{[Ca^{2+}]^{n2} + Kd_2^{n2}}$$

Where Z is the percent change in spectral feature, f_1 and f_2 the fraction of change attributed to the high and low affinity sites, respectively, n_1 and n_2 the Hill coefficients, Kd_1 and Kd_2 the apparent dissociation constants of the low and high affinity sites respectively.

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CHAPTER III

PHOTOCHEMICAL CROSSLINKING BETWEEN NATIVE RABBIT SKELETAL TROPONIN C AND BENZOYBENZOYL-TROPONIN I INHIBITORY PEPTIDE, RESIDUES 104-115

ABSTRACT

The troponin I (TnI) inhibitory region (104-115) was synthesized with Gly-104 labeled [α-^{14}C] and a covalently linked benzoylbenzoyl (BB-) moiety at the N-terminus to yield a photoactivatable radioactive peptide (BBIp). BBIp was crosslinked to rabbit skeletal muscle troponin C (TnC) to locate the binding site on TnC. The TnC/BBIp mixture was subjected to photolysis in aqueous buffer at pH 7.5 in the presence or absence of Ca^{2+}. A covalent (1:1) crosslinked protein-peptide complex (TnC-BBIp) was isolated in both cases. The crosslinked complex was digested with trypsin and the peptide fragments were separated by reversed-phase HPLC. The radioactive crosslinked peptide was isolated and further characterized by peptide sequencing and mass spectrometry before and after cyanogen bromide cleavage. The results indicated that Met 155 of TnC was crosslinked to the BB moiety of BBIp in either the presence or absence of Ca^{2+}. The biological activity of both the BBIp peptide and the crosslinked TnC-BBIp complex was studied and a model of the TnC-Ip complex was derived using molecular dynamic and energy minimization calculations.

INTRODUCTION

Although it is well documented that the inhibitory region (104-115) interacts with TnC, the region on TnC which interacts with the inhibitory region of TnI is not clearly defined. Several studies have indicated that the TnI peptide interacts with the C-terminal domain of TnC (Weeks and Perry, 1978; Chong and Hodges, 1981, 1982a,b; Leavis and

Gergely, 1984; Dalgarno *et al.*, 1982; Drabikowski *et al.*, 1985; Tao *et al*, 1986; Leszyk *et al.*, 1987,1988; Lan *et al.*, 1989; Van Eyk *et al.*, 1991; Swenson and Fredricksen, 1992). In contrast, other crosslinking data indicated that residues from the regulatory Ca^{2+}-binding site II in the N-terminus of TnC (residues 46-78) formed crosslinks with TnI inhibitory segment residues 92-167 (Leszyk *et al.*, 1990). Kobayashi *et al.* (1991) have shown that a mutant TnC containing a single Cys at residue 57 and modified with 4-maleimidobenzophenone can be crosslinked to the inhibitory region of TnI.

To determine the exact binding site on TnC for the TnI inhibitory peptide (Ip), a photoactivatable radioactive TnI peptide (BBIp) (Table III-1) was synthesized by solid-phase methodology and crosslinked to TnC in the presence and absence of calcium. Based on these crosslinking results, we generated a three dimensional model of the TnC/Ip complex. This work is published by S. M. Ngai and R. S. Hodges in J. Biol. Chem. 269, 2165-2172 (1994).

RESULTS

Photolysis of TnC and BBIp — Photolysis of a mixture of native rabbit skeletal TnC and BBIp (1:3.3 molar ratio) in either Ca^{2+} or Mg^{2+} buffer yielded a covalent TnC/BBIp complex (Fig. III-1, panels B and C). Analysis of the complex by SDS gel electrophoresis indicated that photochemical crosslinking between TnC and the BBIp peptide was complete (insert of Fig. III-1, panel B) and that the complex (as indicated by the horizontal bar in Fig. III-1, panel B) consisted of a 1:1.2 ratio of TnC and BBIp by radioactivity measurement and amino acid analysis. The finding that TnC/BBIp complex could be formed in either Mg^{2+} or Ca^{2+} buffer is consistent with previous studies that TnC is able to neutralize Ip induced inhibition in the acto-S1-TM ATPase assay in either the presence or absence of Ca^{2+} in the Mg^{2+}-ATPase buffer (Talbot and Hodges, 1979,

Table III-1

Amino Acid Sequences of TnI and TnC Peptides[a]

TnI Inhibitory Peptide *104-115* (Ip) :

104 115
Ac-GKFKRPPLRRVR-amide

Cross-linking Radioactive TnI Inhibitory Peptide *104-115* (BBIp) :

104 115
BB-GKFKRPPLRRVR-amide[b]

TnC peptide (residues 89-100) :

89 100
NH2- GKSEEELAECFR-COOH

TnC peptide (residues 91-100) :

91 100
NH2- SEEELAECFR-COOH

TnC peptide (residues 154-159) :

154 159
NH2-MMEGVQ-COOH

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[a] These sequences are from primary sequence of rabbit skeletal troponin I and troponin C (Wilkinson and Grand, 1975, 1978)

[b] The TnI inhibitory peptide (Ip) was synthesized with Gly-104 labeled [α-^{14}C] and covalently linked to a benzoylbenzoyl moiety (BB) to yield the BBIp peptide

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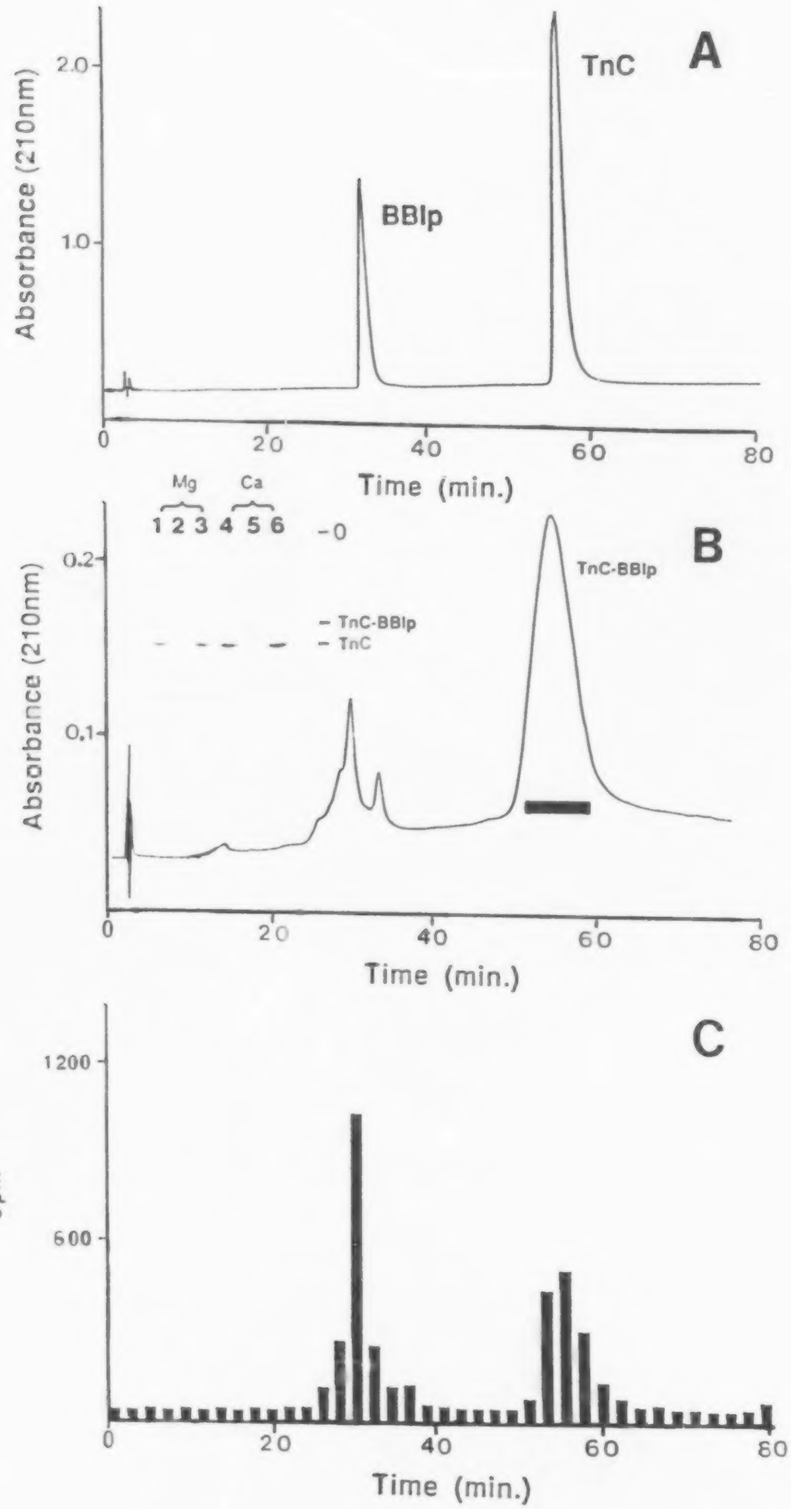
Fig. III-1. **Photolysis Experiment.** Panel A, an aliquot of the non covalent mixture of TnC and BBIp (TnC/BBIp 1:3.3 molar ratio) before photolysis was chromatographed on an Aquapore reversed-phase column (1% acetonitrile per min., 1 ml/min.). Panel B, an aliquot of the same mixture after photolysis was loaded onto the identical Aquapore reversed-phase column under the same running condition (see insert for comparing the position of TnC and the covalent crosslinked TnC/BBIp on SDS gel electrophoresis: lane 1, TnC/Mg before photolysis; lane 2, TnC/BBIp covalent crosslinked complex/Mg; lane 3, TnC/Mg after photolysis; lane 4, TnC/Ca before photolysis; lane 5, TnC/BBIp covalent crosslinked complex/Ca; lane 6, TnC/Ca after photolysis). Panel C, radioactivity trace (collected in one minute fraction) of the RPC run as in panel B.

A
TnC
BBIp
Absorbance (210nm)
2.0
1.0
0
20
40
60
80
Time (min.)
B
Mg
Ca
1 2 3 4 5 6
-0
TnC-BBIp
- TnC-BBIp
- TnC
Absorbance (210nm)
0.2
0.1
0
20
40
60
80
Time (min.)
C
Cpm
1200
600
0
20
40
60
80
Time (min.)

1981a, 1981b; Cachia *et al.*, 1983, 1986; Van Eyk and Hodges, 1987, 1988; Ngai and Hodges, 1992).

Acto-S1-TM ATPase Assay — The relative acto-S1-TM ATPase activity as shown in Fig. III-2 was measured as a function of added protein (TnC, TnC-BBIp, covalent complex and TnC/BBIp non covalent complex) or peptide (Ip, BBIp). In agreement with previous studies in our laboratory, the TnI inhibitory peptide (Ip) inhibited the acto-S1-TM ATPase activity by 72%. Similarly, the BBIp peptide is also capable of inhibiting the acto-S1-TM ATPase activity (80%). This result indicates that the addition of the BB-moiety does not alter the biological function of Ip in the ATPase assay. However, when BBIp is pre-incubated with TnC to form a non-covalent complex, this complex is no longer able to inhibit the acto-S1-TM ATPase activity (Fig. III-2). Similarly, the isolated covalent TnC/BBIp complex isolated after photolysis was not capable of inhibiting the acto-S1-TM ATPase activity. TnI or TnI inhibitory peptide inhibits the acto-S1-TM ATPase activity by interacting with the actin-TM complex, thus, preventing S1 interaction. This result implies that the BBIp peptide once bound non-covalently or covalently to TnC is not capable of simultaneous interaction with the actin-TM complex (results are identical in Ca^{2+} or Mg^{2+} ATPase buffer).

Identification of the site of crosslinking — Complete tryptic digestion of the TnC/BBIp complex (in the presence of Mg^{2+} or Ca^{2+}) was carried out and analyzed by reversed-phase HPLC. Fig. III-3, panel A is the radioactivity trace of the reversed-phase HPLC run of the complete tryptic digest of the TnC/BBIp complex in the presence of Ca^{2+} (panel B). The elution profiles of the tryptic digest were essentially identical for the complex obtained after photolysis in either the presence of Ca^{2+} or Mg^{2+} (absence of Ca^{2+}). The major radioactive fraction (at 37 min.) in Fig. III-3, panel A represents the crosslinked tryptic fragment. Sequence results of this fraction (+Ca^{2+}) indicated the presence of three TnC peptides: residues 154-159, residues 91-100 and residues 89-100

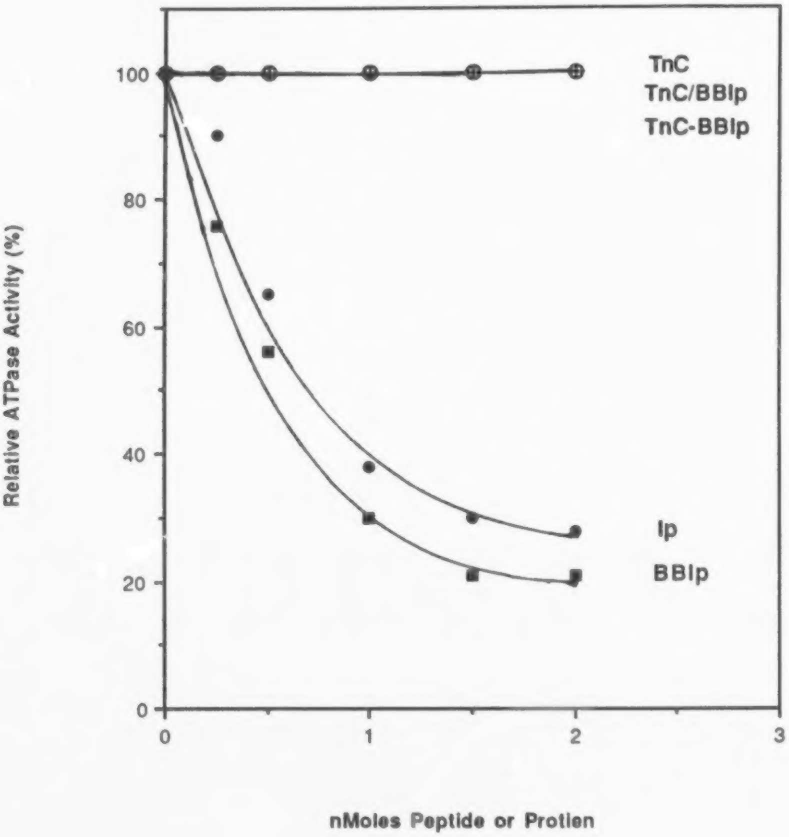


Fig. III-2. **Effect of Ip, BBIp, TnC, TnC/BBIp and TnC-BBIp on the acto-S1-TM ATPase Activity.** The S1, actin and TM concentrations were 3, 1.5 and 0.22 μM, respectively, giving a molar ratio of 14:7:1. (●) Ip; (■) BBIp; (○) TnC; (☐) non covalent TnC/BBIp complex obtained from mixing TnC and BBIp in a 2:1 molar ratio, respectively; (+) covalent TnC-BBIp complex after photolysis.

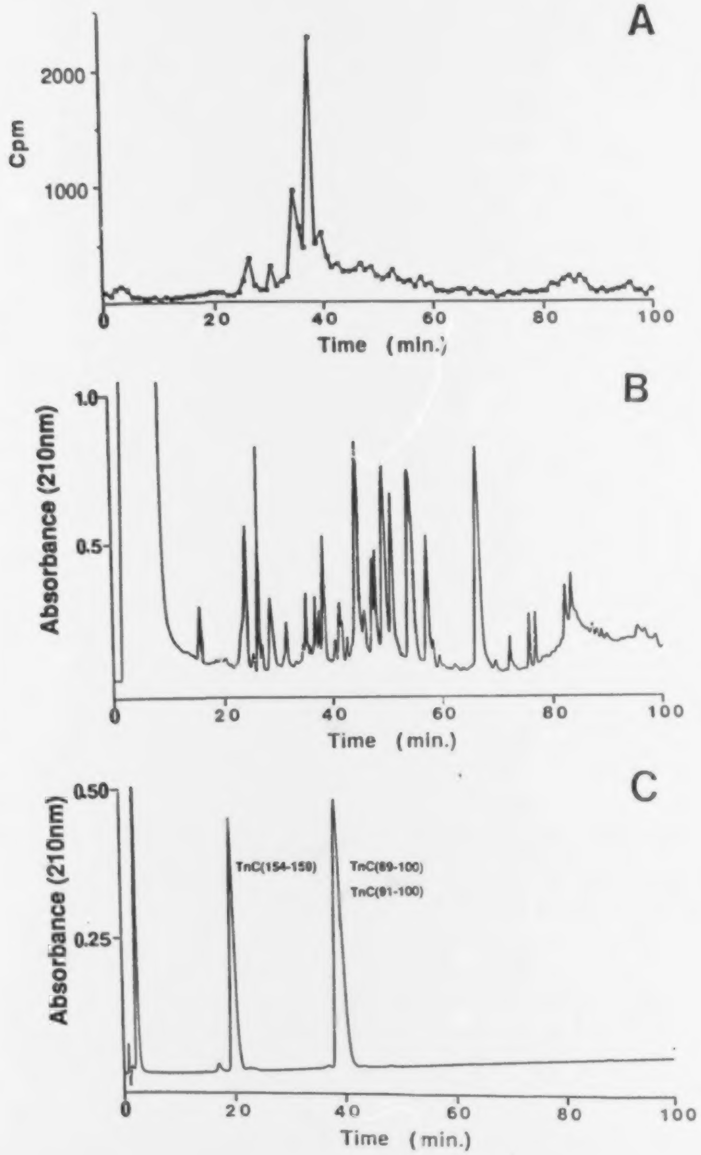


Fig. III-3. **Complete Tryptic Digest of TnC/BBIp complex.** Panel A, radioactivity trace from the RPC run after complete tryptic digestion of the TnC/BBIp complex (see panel B). Panel B, RPC run of the complete tryptic digest of TnC/BBIp complex (0.5% acetonitrile per min., 1 ml/min.). Panel C, RPC run comparing the retention time of the native TnC fragments (residues 154-159, residues 89-100 and residues 90-100) under the identical running condition as in panel B.

(Table III-2, top panel) and only two TnC peptides (-Ca^{2+}) : residues 154-159 and residues 91-100 (Table III-2, bottom panel). In both cases, TnC residues 154-159 is the major sequence that was detected.

The elution profile for the reversed-phase HPLC run of the three synthetic TnC fragments corresponding to residues 154-159, 91-100, and 89-100 (see Table III-1 for sequences) is shown in Fig. III-3, panel C. A comparison with Fig. III-3, panel A and B, shows that the two TnC fragments (89-100 and 91-100) co-elute at the same retention time as the major radioactive peak. However, native TnC 154-159 elutes about 20 min. before the radioactive peak. This is very strong evidence that TnC 154-159 was crosslinked to the BB-G-K moiety which would be obtained from tryptic digestion of TnC/BBIp complex and would change the retention time of TnC 154-159 by increasing the hydrophobicity of the peptide. On the other hand since the radioactive crosslinked peptide has the same retention time as TnC 89-100 or 91-100 suggests that the BB-G-K moiety was not crosslinked to these peptides. Further evidence indicating that TnC 154-159 was crosslinked to BB-G-K comes from the mass spectra of the radioactive fractions obtained from the digest of TnC/BBIp (in the presence of Ca^{2+} or Mg^{2+}) which gave a mass corresponding to the summation of TnC 154-159 and BB-G-K moiety. An interesting observation from the sequencing data was that in both cases a very low sequencer yield of Met 155 was obtained (Table III-2). Since there is no problem with the recovery of PTH-Met from the sequencing of the native peptide, this suggests that this residue was crosslinked to the BB-G-K moiety . To provide further documentation of this proposal, the radioactive fraction was further characterized by cyanogen bromide cleavage. Mass spectrometry data of the CNBr digest (Fig. III-4) indicated that the original TnC 154-159-BB-G-K crosslinked fragment had vanished. However, two fragments, Met-Homoserine Lactone-BB-G-K corresponding to residues 154-155 of TnC and TnC 155-159-BB-G-K

Table III-2

Sequence Analysis of the Radioactive Fraction

Ca^{2+}-Buffer[a]

Cycle #	TnC 154-159 Residue Identified	Yield (pmoles)	TnC 89-100 Residue Identified	Yield (pmoles)	TnC 91-100 Residue Identified	Yield (pmoles)
1	Met	158.37	Gly	38.38	Ser	5.16
2	Met	36.01	Lys	20.17	Glu	8.03
3*	Glu	(90.40)	Ser	N.D.	Glu	(90.40)
4*	Gly	142.57	Glu	(33.62)	Glu	(33.62)
5	Val	159.16	Glu	23.01	Leu	11.20
6**	Gln/Glu	47.46/(28.41)	Glu	(28.41)	Ala	10.28
7			Leu	18.26	Glu	16.19
8			Ala	17.02	Cys	N.D.
9			Glu	8.30	Phe	6.56
10			Cys	N.D	Arg	N.D.
11			Phe	N.D.		
12			Arg	N.D.		

Mg^{2+}-Buffer[a]

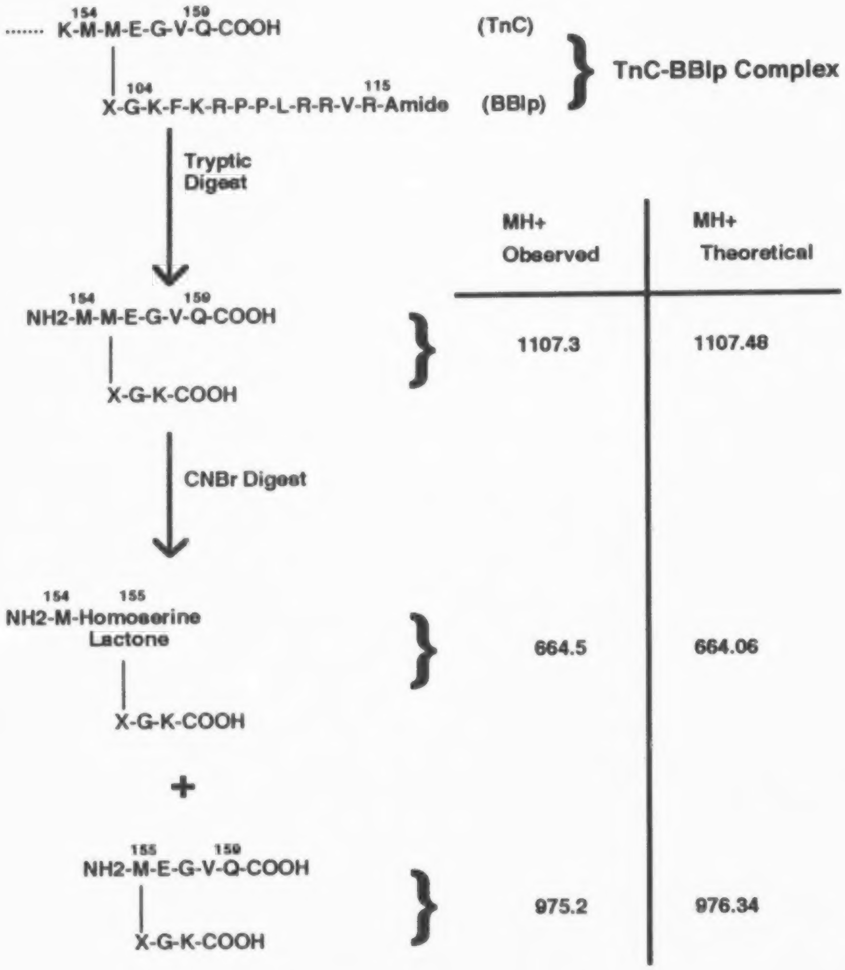
Cycle #	TnC 154-159 Residue Identified	Yield (pmoles)	TnC 91-100 Residue Identified	Yield (pmoles)
1	Met	108.43	Ser	14.80
2	Met	23.66	Glu	33.22
3*	Glu	(94.15)	Glu	(94.15)
4	Gly	98.90	Glu	42.65
5	Val	109.83	Leu	33.96
6**	Gln/Glu	43.83/20.63	Ala	36.04
7			Glu	N.D.
8			Cys	N.D.
9			Phe	26.10
10			Arg	8.55

[a] Sequencing of the radioactive tryptic fragment obtained from the covalent complex formed after photolysis in the presence or absence of Ca^{2+}

N.D. means not determined by sequencer

* Fragments contain a common amino acid at that particular cycle and the value in brackets is the total Glu for two peptides in the mixture

** Gln could be partly deaminated to yield Glu during sequencing



154 159
.... K-M-M-E-G-V-Q-COOH
(TnC)
104 115
X-G-K-F-K-R-P-P-L-R-R-V-R-Amide
(BBIp)
TnC-BBIp Complex
Tryptic Digest
MH+ Observed
MH+ Theoretical
154 159
NH2-M-M-E-G-V-Q-COOH
X-G-K-COOH
1107.3
1107.48
CNBr Digest
154 155
NH2-M-Homoserine Lactone
X-G-K-COOH
664.5
664.06
+
155 159
NH2-M-E-G-V-Q-COOH
X-G-K-COOH
975.2
976.34

Fig. III-4. **Summary for Characterization of the TnC/BBIp Complex.** Results are similar in either the presence or absence calcium and X denotes the benzoylbenzoyl moiety. See Materials and Methods for experimental conditions.

were observed. From the above experimental results, we concluded that Met-155 of TnC was crosslinked to the BB moiety of the BBIp peptide.

Three Dimensional Computer Modeling — Initial coordinates of the C-terminal domain were obtained from the X-ray crystal structure of turkey TnC (Herzberg and James, 1988), which has an identical sequence in this region to rabbit skeletal TnC. As starting coordinates for the Ip peptide, the NMR-derived structure of Ip bound to TnC (Campbell and Sykes, 1991; Campbell *et al.*, 1991) was used. To determine the preferred position of the peptide in the hydrophobic pocket of the C-terminal domain a procedure consisting of alternately docking the rigid peptide to the rigid protein and optimization of sidechain conformations in the complex by energy minimization was employed. The minimization step was required in order to allow the generation of an "induced fit" between protein and ligand since the crystal structure of TnC was obtained in absence of a ligand, and moreover, since the structure of the bound Ip-peptide showed only a well-defined backbone conformation and could not define the orientations of sidechains. In particular, positions of Lys and Arg sidechains were not refined due to the degeneracy of their proton resonances, which caused significant signal overlap in the transferred NOE-experiments and prevented the determination of inter atomic distances involving these sidechains.

The initial placement of the Ip-peptide in the hydrophobic pocket made use of the above crosslinking results by placing the N-terminus of Ip in close proximity of Met 155 of TnC, which at the same time fixes the relative orientation of the peptide in the groove of TnC. The modeled complex-structure was nearly independent of the initial placement as shown by repeating the calculations with several different starting coordinates. This was also confirmed by independent calculations (Slupsky and Sykes, personal communication).

In the derived model of the complex (Fig. III-5, panel B) the Ip-peptide is situated in the hydrophobic pocket of the C-terminal domain of TnC with the N-terminus close to

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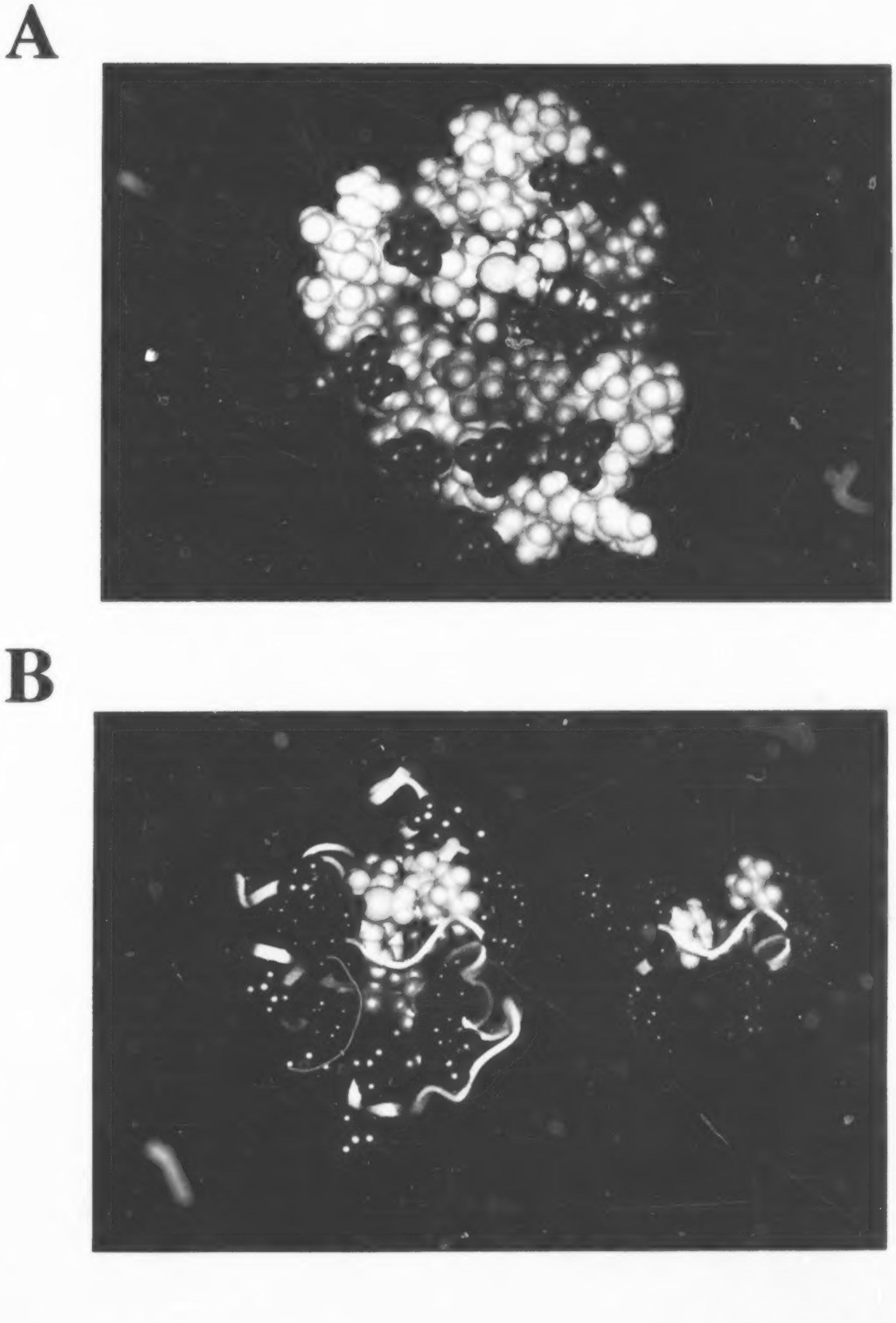
Fig. III-5. **Molecular modeling**. Panel A, space filling model of TnC C-domain. The side chains are colored as follows: Cys 98 (yellow); Met 154, 155 (yellow); Phe 102, 154 (brown); Leu, 119, 134 (light blue); Val 126 (light green); Ile 115, 118 (dark green); Asp and Glu (red); polypeptide backbone and residues not involved in binding are colored white. These colored residues are involved in the binding of Ip in the C-domain-Ip complex (panel B). Panel B (left), C-domain-Ip complex (TnC, polypeptide backbone in white ribbon; Ip, polypeptide backbone in pink ribbon). The view of the C-domain is identical to that shown in panel A and Ip is lying on top of the groove of C-domain. Panel B (right), horizontal displacement of Ip from the C-domain-Ip complex (left). The colored side chains of Ip are those involved in the binding to the C-domain of TnC (side chains are colored as follows: Lys and Arg (blue); Phe 106, (brown); Leu 111 (light blue); Val 114 (light green)).


A
B

Met-155 of the H-helix of TnC. The complex shows favorable interactions between several residues, leading to a negative intermolecular VDW-energy as well as negative intermolecular Coulomb energy. The RMS-deviations for backbone atoms in the complex and in the initial structures are small for protein and peptide, indicating that no backbone conformational changes were required to obtain a favorable position of the bound peptide. The N-terminus of Ip stayed within 5 Å of Met 155; a distance suitable for crosslinking to the BB-moiety. Hydrophobic contacts were observed between Phe 106 and Val 114 of Ip and Phe 102, Ile 118, Leu 119, Leu 134, Phe 151, Met 154 and Met 155 of TnC. Many of these residues have been shown to be perturbed upon binding of Ip to the C-terminal domain of TnC (formed by two peptides representing Ca^{2+}-binding sites III and IV) by NMR spectroscopy (Slupsky *et al.*, 1992). Electrostatic interactions (Glu and Asp (red sidechains) from TnC and Lys and Arg (blue sidechains) from Ip) were found as indicated in Fig. III-5, panel B (left). An analysis of the solvent accessible surface areas of TnC and Ip alone and in the TnC/Ip complex underline the complementary fit even further (Table III-3). The hydrophobic residues Phe 106 and Val 114 of Ip and Phe 102, Leu 134, Met 154 and Met 155 of TnC become completely buried in the complex (fractional area accessible is less than 0.1) and had a change of more than 50 $Å^2$ in solvent accessible area. Additional polar or charged residues showing a decrease of greater than 50 $Å^2$ in solvent accessible area upon complex formation were Thr 122 and Glu 124 in TnC and Lys 107, Arg 113 and Arg 115 in Ip (Table III-3). Overall, complex formation reduces the surface of TnC and Ip by 1259 $Å^2$, of which 818 $Å^2$ are nonpolar surface areas. For TnC/Ip complex compared to TnC this is equivalent to a net decrease of 120 $Å^2$ in non polar and an increase in polar and charged surface area by 254 and 475 $Å^2$, respectively (Table III-4).

DISCUSSION

Chemical crosslinking studies have been used extensively in an attempt to determine the binding interface between TnC and TnI (Chong and Hodges, 1981, 1982a,b; Tao *et al.*,

Table III-3

Solvent accessible area of residues in TnC, Ip or TnC/Ip complex[a]

residue:	area (Å^2)	fractional area	area (Å^2)	fractional area
	TnC (free)		TnC (in complex)	
CYS 98	37.0	0.26	19.2	0.13
(PHE 102)[b]	52.5	0.23	2.1	0.01
ILE 118	19.4	0.10	0.1	0.00
LEU 119	39.0	0.19	15.2	0.07
ALA 121	93.9	0.76	80.7	0.65
(THR 122)	93.2	0.62	25.9	0.17
(GLU 124)	132.1	0.70	67.3	0.35
ASP 130	71.1	0.45	52.9	0.34
(LEU 134)	76.7	0.37	8.8	0.04
ASP 137	108.7	0.69	87.1	0.55
(MET 154)	59.0	0.27	3.6	0.02
(MET 155)	93.6	0.43	16.4	0.08
GLU 156	110.8	0.58	91.2	0.48
VAL 158	163.8	0.95	143.5	0.84
	Ip (free)		Ip (in complex)	
GLY 104	57.2	0.63	26.8	0.29
LYS 105	180.8	0.84	136.5	0.64
(PHE 106)[b]	155.4	0.69	5.7	0.02
(LYS 107)	202.3	0.94	11.8	0.52
LEU 111	133.5	0.64	91.9	0.44
ARG 112	204.6	0.84	185.9	0.76
(ARG 113)	196.7	0.81	121.5	0.50
(VAL 114)	56.7	0.33	1.5	0.01
(ARG 115)	255.2	1.05	163.7	0.67

---

[a] Residues are listed only if their fractional accessible surface area changes by ≥ 0.10 between free peptide/ protein and the modelled complex structure.

[b] Brackets indicate residues whose solvent accessible area in the complex deceased by more than 50 Å^2 from the free state.

Table III-4

Summary of Solvent Accessible Surface Areas (ASA , Å^2)

	TnC	Ip	(TnC+Ip)	TnC/Ip Complex
Nonpolar ASA	2187.2	698.8	2886.0	2067.7
Polar ASA	1470.3	505.9	1976.2	1724.0
Charged ASA	1523.2	663.9	2187.1	1998.5
Total ASA	5180.7	1868.6	7049.3	5790.2

1986; Leszyk *et al.*, 1987, 1988 and 1990; Kobayashi *et al.*, 1991). Results have demonstrated that Cys-98 of TnC can be crosslinked using a photoreactive crosslinker (4-maleimidobenzophenone) to TnI in the inhibitory region 103-110 (Leszyk *et al.*, 1987). On the other hand, Leszyk *et al.* (1990) indicated interaction (using zero crosslinking reagents) between Site II of TnC with the inhibitory region of TnI and Kobayashi *et al.* (1991) demonstrated that a mutant TnC containing a single Cys at residue 57 and modified with 4-maleimidobenzophenone can be crosslinked to TnI in the region 113-121. These results suggest that both the N- and C-terminal domains of TnC are in close proximity to the inhibitory region (residues 104-115) of intact TnI and support the conclusion that TnC in the presence of TnI adopts a more compact conformation in benign solution (Wang *et al.*, 1987 and Cheung *et al.*, 1991) than in the crystal structure of TnC (Sundaralingam *et al.*, 1985; Herzberg and James, 1985). The crystal structure of TnC shows two structurally independent domains linked via a long central helix (the N-terminal domain contains the two low affinity Ca^{2+}-specific binding sites I and II and the C-terminal domain contains the two high affinity Ca^{2+}/Mg^{2+} binding sites III and IV). The N and C-terminal globular domains of TnC have large hydrophobic pockets (Kretsinger *et al.*, 1986 and Babu *et al.*, 1988) flanked by regions of negatively charged acidic residues (Fig. III-5A), perfectly constructed as binding sites for positively charged basic, amphiphilic helices (similar to a hydrophobic core surrounded by positively charged amino acids). There is evidence to suggest that Ca^{2+} binding to TnC results in increased exposure of these hydrophobic patches, which then become the binding sites for target molecules (La Porte *et al.*, 1980; Gariepy and Hodges, 1983; Cachia *et al.*, 1985).

The TnI inhibitory peptide, Ip (residues 104-115), carries the minimum sequence necessary for inhibition of acto-S1-TM ATPase activity (Talbot and Hodges, 1979; Cachia *et al.*, 1983,1985; Van Eyk *et al.*, 1991). Ip has four arginines and two lysines within a twelve residues sequence. There are hydrophobic residues (phenyalanine, leucine and valine) alternating with these basic residues (Fig. III-5, panel B, left). In our laboratory, Van Eyk and Hodges, 1988 and Van Eyk *et al.* 1991 have evaluated the contribution of each amino acid residue of the TnI inhibitory peptide (Ip) and have shown that the most important residues for binding and inhibitory activity were F106, R108, L111, R113, V114 and R115. The structure of Ip bound to calcium-saturated rabbit skeletal TnC, derived from two-dimensional transferred Nuclear Overhauser effect ^{1}H NMR spectroscopy, reveals an amphiphilic structure, distorted in the center by the two proline residues (Campbell and Sykes, 1989, 1991; Campbell *et al.*, 1991). The central bent structure of the peptide provides the residues on the hydrophobic interface in close proximity with each other to form a hydrophobic knob. The hydrophilic, basic residues extend off the opposite face of the peptide (Fig. III-5, panel B). This Ip structure (hydrophobic knob) fits perfectly onto the hydrophobic groove of TnC.

The C-domain of TnC is structurally homologous to both the N- and C-domains of calmodulin. The binding of Ip to TnC is similar to the binding of the myosin light chain kinase (MLCK) peptide (residues 1-20) to calmodulin (CaM). Recently, the structure of the CaM-MLCK peptide complex was studied and demonstrated the above complementary fit based on a NMR study of interproton distances between the peptide and the N- and C-domains of CaM (Ikura *et al.*, 1992). Slupsky and Sykes (personal communication) used the position of the MLCK-peptide on CaM to model a TnC/Ip complex by homology which resulted in a position of Ip in the C-terminal groove of TnC similar to the one obtained in this study. Three dimensional X-ray scattering studies also demonstrated that TnI (96-115), which is an extended version of the TnI inhibitory peptide (Ip), is able to bind to TnC and break the dimerization interaction of TnC although the binding of the peptide to TnC does not induce a major conformational change in TnC (Blechner *et al.*, 1992). Strynadka and James (1991) predicted that dimerization of TnC involves the interaction of the apolar face of a helix from one TnC molecule into the exposed hydrophobic cleft of a second TnC molecule with electrostatic interaction being formed by negatively charged groups that surround the hydrophobic cleft and positively charged groups from the corresponding helix of the TnC molecules.

Our results showed that peptide binding was very specific in either Mg^{2+}- or Ca^{2+}-buffer since only a single major crosslinked TnC fragment (residues 154-159) was detected. This gives us a valuable constraint parameter in constructing the three dimensional TnC/Ip complex. The resulting structure of the complex (Fig. III-5, panel B) indicates that TnC Cys-98 is in close proximity (within 5 Å) to the Ip peptide which would be crosslinked via a crosslinker at TnC Cys-98 position (Chong and Hodges, 1981, 1982a; Tao *et al.*, 1986 and Leszyk *et al.*, 1987). TnC Phe-102 and Phe-151 are participating in the binding interface within the complex, which is in agreement with the observation from fluorescence studies on recombinant TnC mutant (Phe-102 or Phe-151 was replaced by Trp) and TnC domains (Phe-102 was replaced by Trp). These studies indicated that the preferred binding site for the TnI inhibitory peptide was the C-terminal domain of TnC in either Mg^{2+} or Ca^{2+} buffer (Smillie *et al.*, unpublished results).

We have recently demonstrated that the N-terminal TnI peptide TnI1-40, Rp (residues 1-40), when bound to TnC prevents Ip from interacting with TnC (Ngai and Hodges, 1992) and strongly interacts with C-terminal domain of TnC (Ngai and Hodges, unpublished data). The switching between the TnI peptides (Rp and Ip) within the hydrophobic groove of TnC C-domain reveals that Rp is a negative effector of the regulatory process. Wang *et al.* (1990) demonstrated that binding of cations to the high affinity sites (sites III and IV) of a mutant TnC altered the environment around the amino acid at position 57 in the N-terminal domain (sites I and II). Grabarek *et al.* (1986) and Rosenfeld and Taylor (1985) showed that the binding of Ca^{2+} to the low affinity sites altered the environment around Cys 98 in the C-terminal domain. These findings indicated that the N and C-terminal domains of TnC are communicating with each other upon the binding of Ca^{2+} or Mg^{2+}. Therefore, there must be a modulating event which governs the release of the TnI inhibitory region (Ip) region from TnC by the Rp region of TnI. Although the N-domain of TnC contains the Ca^{2+}- regulatory sites that control muscle contraction, the two most important biologically active regions of TnI (Ip and Rp) bind to the C-domain. This raises the question of how Ca^{2+}- binding to the N-domain of TnC transmits this information to the C-domain of TnC.

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CHAPTER IV

BIOLOGICALLY IMPORTANT INTERACTIONS BETWEEN SYNTHETIC PEPTIDES OF THE N-TERMINAL REGION OF TnI AND TnC

ABSTRACT

The interaction between troponin I and troponin C plays a critical role in the regulation of muscle contraction. In this study the interaction between TnC and the N-terminal region of TnI was investigated by the synthesis of three TnI peptides (residues 1-40/Rp, 10-40 and 20-40). The regulatory peptide, Rp, on binding to TnC prevents the ability of TnC to release the inhibition of the acto-S1-Tm ATPase activity caused by TnI or the TnI inhibitory peptide (Ip), residues 104-115. A stable complex between TnC and Rp in the presence of Ca^{2+} was demonstrated by polyacrylamide gel electrophoresis in the presence of 6M urea. Rp was able to displace TnI from a preformed TnI-TnC complex. In the absence of Ca^{2+}, Rp was unable to maintain a complex with TnC in benign conditions of polyacrylamide gel electrophoresis which demonstrates the Ca^{2+}-dependent nature of this interaction. Size-exclusion chromatography demonstrated that the TnC-Rp complex consisted of a 1:1 complex. The results of these studies have shown that the N-terminal region of TnI (1-40) plays a critical role in modulating the Ca^{2+}-sensitive release of TnI inhibition by TnC.

INTRODUCTION

In the present study, we have focused on the study of N-terminal region of TnI. Synthetic peptides corresponding to the fragments of the N-terminus (1-40/Rp, 20-40, 10-40) and C-terminus (104-115/Ip) of TnI (see Table IV-1 for peptide sequences) were synthesized by solid-phase methodology and used to investigate the biologically important

Table IV-1

Amino Acid Sequences of TnI Peptides [a]

TnI Inhibitory Peptide 104-115 (Ip)

104 115
Ac-GKFKRPPLRRVR-amide

TnI N-terminal Peptides

1 10 20 30 40
NH_2-GDEEKRNRAITARRQHLKSVMLQIAATELEKEEGRREAEK-amide
Rp (TnI 1-40)

10 20 30 40
Ac-ITARRQHLKSVMLQIAATELEKEEGRREAEK-amide
(TnI 10-40)

20 30 40
Ac-VMLQIAATELEKEEGRREAEK-amide
(TnI 20-40)

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[a] The sequence of rabbit skeletal (fast muscle) troponin I (Wilkinson *et al.*, 1975; 1978).

interactions between TnC and the N-terminal region of TnI. This work is published by S. M. Ngai and R. S. Hodges in J. Biol. Chem. 267, 15715-15720 (1992).

RESULTS

Effect of TnI N-terminal Regulatory Peptide 1-40 (Rp) and Truncated TnI Analogs, TnI (10-40) and TnI (20-40) on the Acto-S1-TM ATPase Activity — The relative acto-S1-TM ATPase activity (Fig. IV-1) was measured as a function of added protein (TnI) or peptide (Rp and Ip). In agreement with previous studies in our laboratory, both TnI and TnI inhibitory peptide 104-115 (Ip) inhibited the acto-S1-TM ATPase activity to 15% and 28%, respectively. The TnI N-terminal regulatory peptide 1-40 (Rp) had no inhibitory effect on the acto-S1-TM ATPase activity. The other truncated TnI analogs (TnI 10-40 and TnI 20-40) also showed no inhibitory effect . This observation suggests that the TnI N-terminal peptides are not capable of binding to the actin-tropomyosin complex and do not interfere in any way with the S1-actin interaction responsible for the ATPase activity. Centrifugation studies (Table IV-2) indicate that Rp remains in the supernatant and does not bind to actin-TM which is found in the pellet. This result is in agreement with that reported by Syska *et al.*, (1976) that the CF2 fragment (residues 1-47) of rabbit skeletal TnI did not inhibit the ATPase activity or bind to a G-actin affinity column.

Effect of Rp and Truncated TnI Analogs, TnI(10-40) and TnI(20-40) on TnC Release (in the Presence and Absence of Ca^{2+}) of the Acto-S1-TM ATPase Inhibition by TnI or TnI Inhibitory Peptide (Ip) — The acto-S1-TM ATPase activity was inhibited with either TnI or the TnI inhibitory peptide (Ip), followed by the release of inhibition by TnC in the presence of Ca^{2+} and in the absence or presence of TnI N-terminal peptides [Rp, TnI(10-40) and TnI(20-40)], as shown in Fig. IV-2. In the presence of Ca^{2+}, TnC released the inhibition induced by either TnI or Ip. The addition of the N-terminal TnI peptides prevented TnC from fully releasing the inhibition induced by TnI or TnI inhibitory

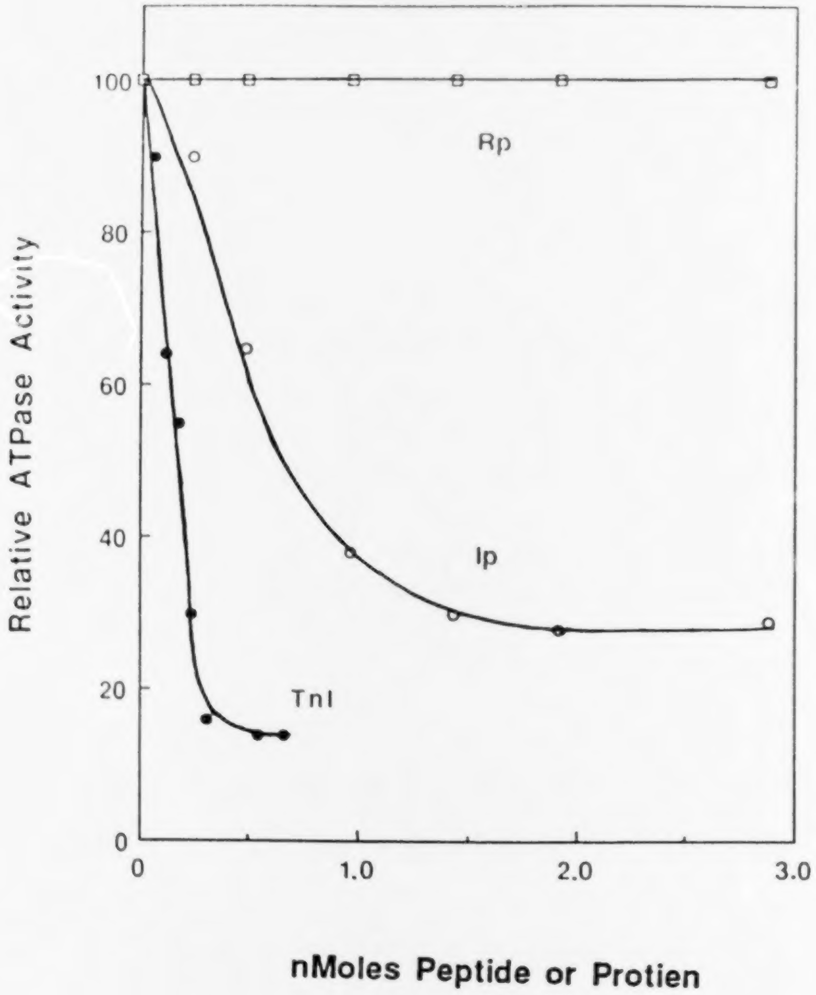
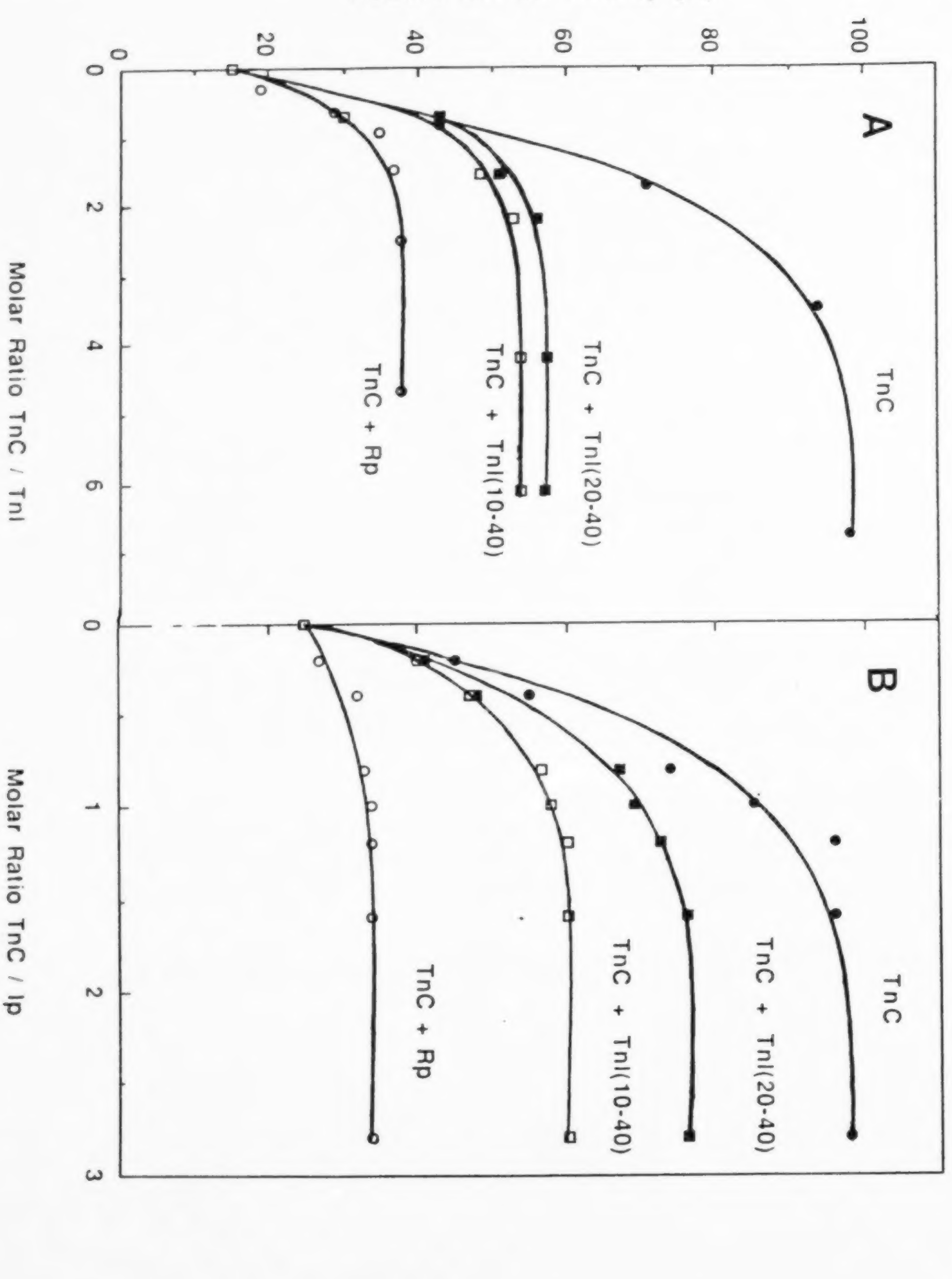


Fig. IV-1. **Effect of TnI and TnI peptides on the acto-S1-TM ATPase Activity.** The S1, actin and TM concentrations were 3, 1.5 and 0.22 μM, respectively, giving a molar ratio of 14:7:1. (●) TnI; (○) Ip; (□) Rp.

Fig. IV-2. **Effect of N-terminal TnI peptide on TnC (Ca^{2+}) in release of acto-S1-TM ATPase inhibition by TnI or TnI inhibitory peptide (Ip).** The S1, actin and TM concentrations were 3, 1.5 and 0.22 μM, respectively, giving a molar ratio of 14:7:1. (●) TnC; (■) TnC + TnI (20-40) [1:2]; (□) TnC + TnI (10-40) [1:2] (○) TnC + Rp [1:1.6]


A
B
Relative ATPase Activity (%)
0
20
40
60
80
100
TnC
TnC + TnI(20-40)
TnC + TnI(10-40)
TnC + Rp
0
2
4
6
Molar Ratio TnC / TnI
0
1
2
3
Molar Ratio TnC / Ip

peptide (Ip) (Fig. IV-2, panel A and B). Rp was most effective in preventing the release of inhibition by TnC (38% activity for TnI as the inhibitor (panel A) and 28% activity for Ip as the inhibitor (panel B)).

Results in Fig. IV-3 (panel A) shows that Rp was more effective in blocking the neutralization of TnI inhibition by TnC in the presence of Ca^{2+} (relative ATPase activity is 100% in the absence of Rp and 38% in the presence of Rp) compared to the absence of Ca^{2+} (relative ATPase activity is 71% in the absence of Rp and 60% in the presence). When Ip was the inhibitor (Fig. IV-3, panel B), the effect of Rp in blocking the neutralization by TnC in the presence of Ca^{2+} (100% activity in the absence of Rp and 34% in the presence of Rp) was similar to that observed when TnI was the inhibitor. In contrast, Rp was more effective in blocking the neutralization of Ip inhibition by TnC in the absence of Ca^{2+} (75% activity in absence of Rp and 23% activity in the presence of Rp, Fig. IV-3B) than TnI inhibition (71% activity in the absence of Rp and 60% in the presence, Fig. IV-3A). The effect of Rp in blocking the neutralization of TnI inhibition by TnC compared to that of blocking the neutralization of Ip inhibition is complicated by the fact that the Rp sequence exists in TnI. Thus, both Rp and the Rp region in TnI could be in competition for TnC. In contrast with Ip inhibition, residues 104-115 of TnI do not overlap with the sequence of Rp, residues 1-40 allowing for easier interpretation of the results. These results suggest that Rp may be preventing the binding of Ip to TnC in the absence and presence of Ca^{2+}. To show that Ip actually binds to actin-TM as indicated by the ATPase results in the absence or presence of Ca^{2+} and that Ip is released from actin-TM by the addition of TnC in a Ca^{2+}-dependent manner, centrifugation studies were performed as shown in Table IV-2.

Determination of the Interaction Between TnI, TnC and Rp by Polyacrylamide Gel Electrophoresis — Head and Perry (1974) first demonstrated the formation of an extremely

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Fig. IV-3. **Effect of Rp (TnI 1-40) on the release of acto-S1-TM inhibition by TnC in the absence or presence of Ca^{2+}.** The S1, actin and TM concentrations were 3, 1.5 and 0.22 μM, respectively, giving a molar ratio of 14:7:1. (●) TnC saturated with Ca^{2+}; (○) TnC without Ca^{2+}; (■) TnC saturated with Ca^{2+} + Rp; (□) TnC without Ca^{2+} + Rp.

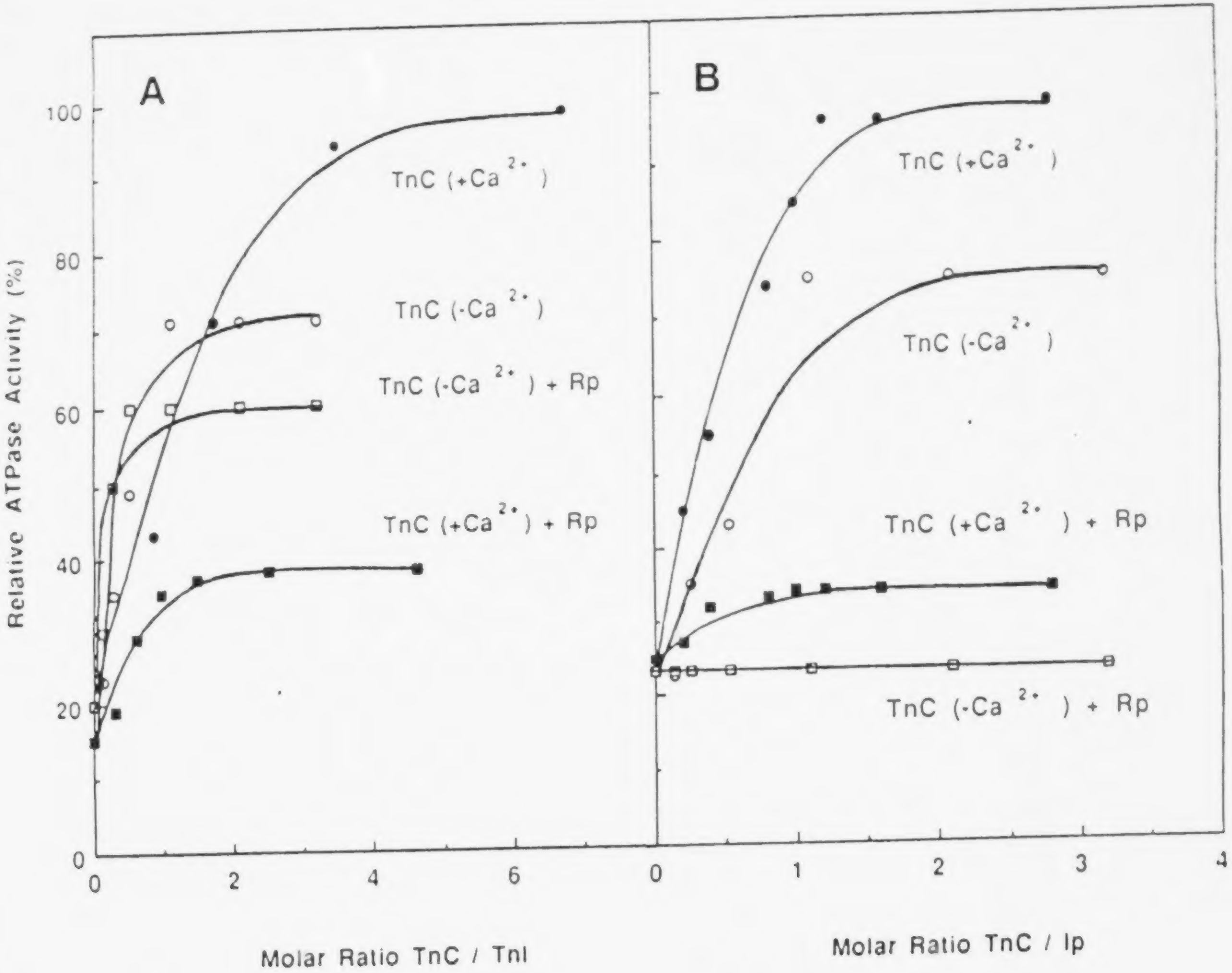

A
B
Relative ATPase Activity (%)
0
20
40
60
80
100
TnC (+Ca $^{2+}$)
TnC (-Ca $^{2+}$)
TnC (-Ca $^{2+}$) + Rp
TnC (+Ca $^{2+}$) + Rp
0
2
4
6
Molar Ratio TnC / TnI
TnC (+Ca $^{2+}$)
TnC (-Ca $^{2+}$)
TnC (+Ca $^{2+}$) + Rp
TnC (-Ca $^{2+}$) + Rp
0
1
2
3
4
Molar Ratio TnC / Ip

Table IV-2

Centrifugation Studies of Ip or Rp Binding With Actin-TM

Components in Assay	Percent Peptide Bound to Actin-TM[a]	
	+ Mg^{2+}	+ Ca^{2+}
Actin-TM-Ip-ATP	51%	55%
Actin-TM-Ip-ATP-TnC	27%	6%
Actin-TM-Rp-ATP	0%	0%
Actin-TM-Rp-ATP-TnC	0%	0%

[a] C^{14}-labelled Ip was used in these binding studies. After the actin-TM was pelleted the C^{14}-labelled Ip in the supernatant was determined. The quantity of Rp in the supernatant was determined by reversed-phase HPLC.

stable IC complex between TnI and TnC in the presence of Ca^{2+} by using polyacrylamide gel electrophoresis. The complex was stable in 6 M urea in the pH range 7.0-8.6.

Analysis of the TnI, TnC and Rp interaction was carried out by the modified method of Head and Perry (see "Chapter II, Materials and Methods"). The results shown in Fig. IV-4 demonstrated that both TnI and Rp were capable of forming a stable complex with TnC (Lane 2 and 4, respectively). Interestingly, Rp was able to displace TnI from the pre-formed TnI/TnC complex (Lane 6) and TnI could not displace Rp from the pre-formed Rp/TnC complex (Lane 8). This strongly agrees with results obtained in the acto-S1-TM ATPase assay where Rp/TnC complex was unable to displace effectively TnI or Ip from its actin-TM binding site. In addition, in the absence of Ca^{2+}, Rp was unable to form complexes with TnC that were stable to polyacrylamide gel electrophoresis (see Fig. V-3 in chapter V) in either benign or denaturing (6M urea) conditions.

Study of the Interaction between Rp and TnC by Size Exclusion Chromatography — The interaction between Rp and TnC in the presence of Ca^{2+} was further studied using HPLC methodology. The top and bottom diagram of Fig. IV-5 (panel A) show the corresponding elution profiles of Rp and TnC on size-exclusion (SEC) and Reversed-phase chromatography (RPC), respectively. Fig. IV-5 (panel B, Top) shows the SEC run of the pre-formed TnC/Rp complex. The complex peak was collected from the size-exclusion run followed by reversed-phase chromatography. TnC and Rp were easily separated on RPC (bottom of panel B). The complex (TnC/Rp) was shown by RPC to consist of a 1.1 : 1 ratio of TnC and Rp (integration of peak areas). These results showed that Rp was capable of forming a stable complex with TnC in the presence of Ca^{2+} during SEC. The presence of a 1:1 complex of Rp with TnC could not be demonstrated in the absence of Ca^{2+} by SEC. The finding that the TnC/Rp complex was not observed in the absence of Ca^{2+} by size-exclusion chromatography or polyacrylamide gel electrophoresis is not inconsistant

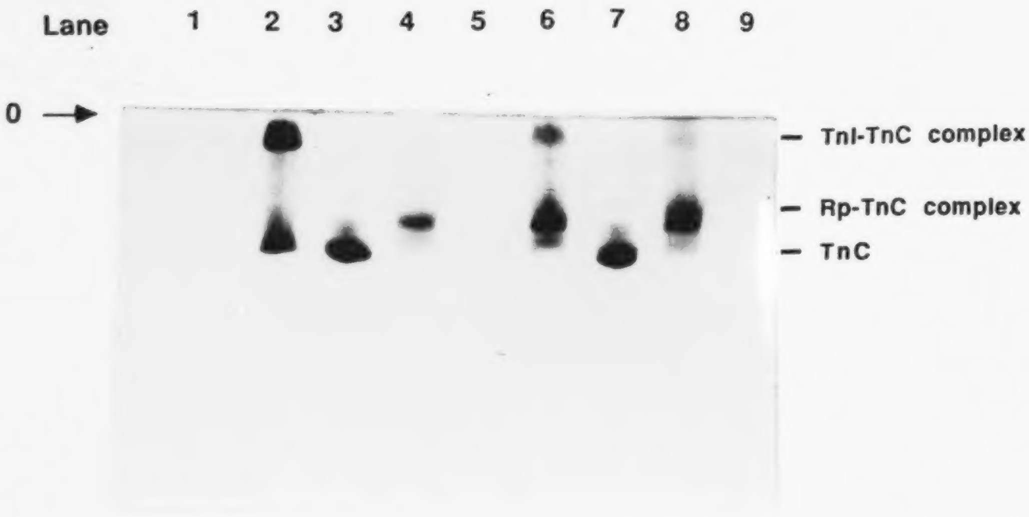
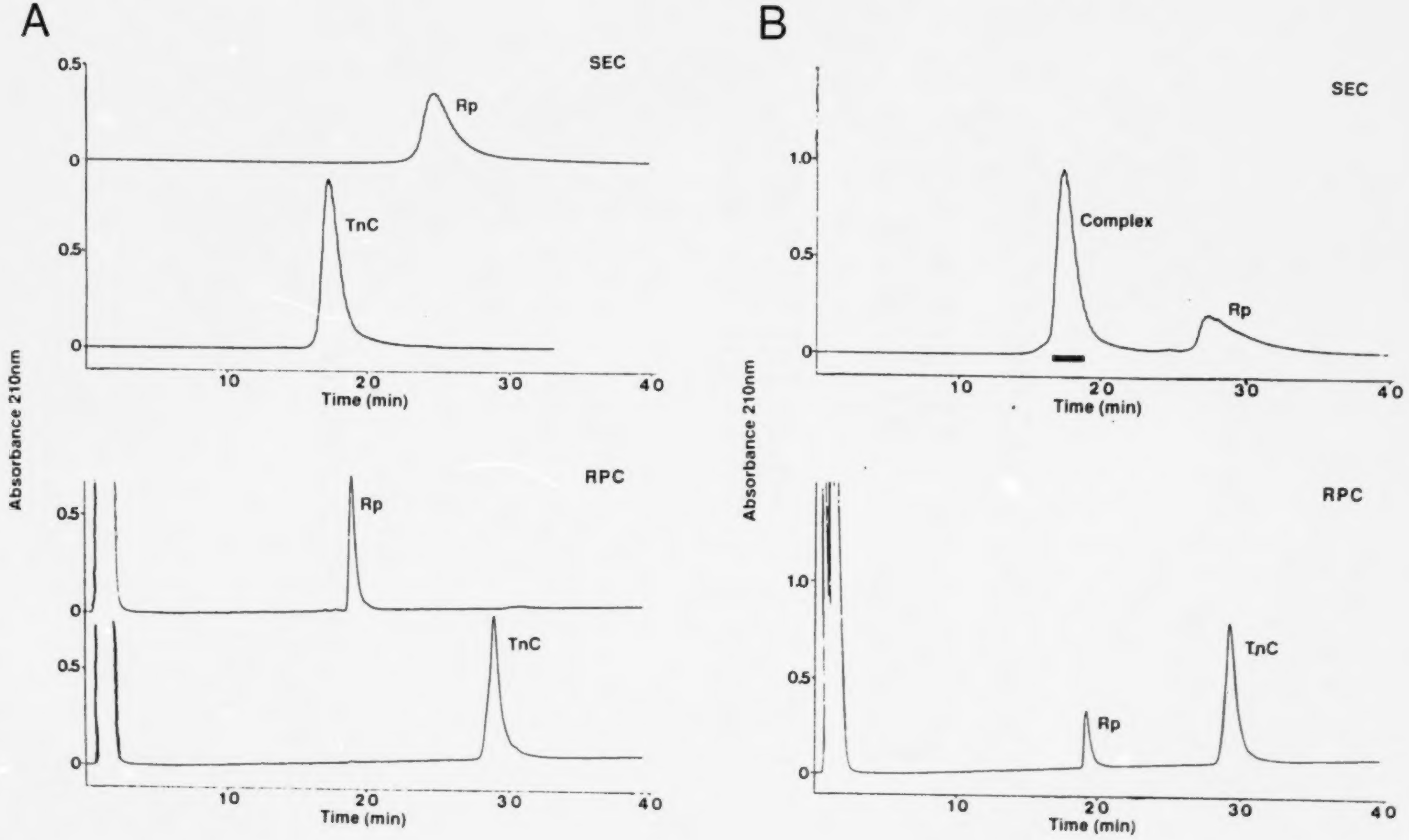


Fig. IV-4. **Polyacrylamide gel electrophoresis.** The polyacrylamide gel (8% crosslinked) was polymerized in a Tris/glycine/6M urea buffer, pH 8.6 with Ca^{2+} (3 mM) and DTT (1mM). The running buffer did not contain urea. Lane 1, TnI (1 nmole); Lane 2, TnI (1 nmole) and TnC (1 nmole) equilibrated for 3 hr; Lane 3, TnC (1 nmole); Lane 4, Rp (2 nmole) and TnC (1 nmole) equilibrated for 3 hr; Lane 5, TnI (1 nmole); Lane 6, TnI (1 nmole) and TnC (1 nmole) equilibrated for 3 hr prior to addition of Rp (2 nmole) [another 3 hr equilibration before loading onto the gel]; Lane 7, TnC (1 nmole); Lane 8, Rp (2 nmole) and TnC (1 nmole) equilibrated for 3 hr prior to addition of TnI (1 nmole) [another 3 hr equilibration before loading onto the gel]; Lane 9, TnI (1 nmole). See Methods section for preincubation condition. TnI stays at origin and is not seen in this gel. In addition, Rp by itself also stays at origin and is not seen in gel (not shown).

Fig. IV-5. **Study of the interaction between Rp and TnC by HPLC.** Panel A (top and bottom), TnC (8 nmoles in 80 μl) and Rp (16 nmoles in 64 μl) were individually chromatographed on a TSK (G2000 SW) size-exclusion column. TnC and Rp peaks were collected and loaded onto a microbore reversed-phase column (bottom of panel A). Panel B (top), TnC (10 nmoles in 100 μl) and Rp (20 nmoles in 80 μl) were preincubated together for 3 hours to form a TnC/Rp complex in the presence of Ca^{2+}. The preincubated mixture (144 μl) was loaded onto the SEC column and the complex (indicated by the horizontal bar) was collected and loaded onto the microbore reversed-phase column (bottom). See Chapter II (Materials and Methods) for conditions of incubation and running buffers for the SEC and RPC runs.


A
SEC
Rp
TnC
0.5
0
0.5
0
10
20
30
40
Time (min)
Absorbance 210nm
RPC
Rp
TnC
0.5
0
0.5
0
10
20
30
40
Time (min)
B
SEC
Complex
Rp
1.0
0.5
0
10
20
30
40
Time (min)
Absorbance 210nm
RPC
Rp
TnC
1.0
0.5
0
10
20
30
40
Time (min)

with the suggestions of a TnC/Rp complex in the absence of Ca^{2+} in the ATPase assays (Fig. IV-3). This can be explained by a large decrease in affinity of Rp for TnC in the absence of Ca^{2+} compared to the presence of Ca^{2+} and further demonstrates the Ca^{2+}-sensitive nature of complex formation between TnC and Rp.

DISCUSSION

Previous investigations on the biological interactions between fragments or synthetic peptides of TnI and the protein TnC or fragments of TnC and the protein TnI (Syska *et al.*, 1976; Weeks and Perry, 1978; Leavis *et al.*, 1978; Talbot and Hodges, 1979, 1981a, 1981b; Evans and Levine, 1980; Nozaki *et al.*, 1980; Grabarek *et al.*, 1981; Katayama & Nozaki, 1982; Leavis and Gergely, 1984 (review); Van Eyk and Hodges, 1988) demonstrated that TnI contains in its primary structure, two regions (1-47 and 104-115) that can interact with TnC, and TnC contains three regions (residues 49-61, 89-100 and 127-138) that interact with TnI. However, these sites of interaction may be even more complex than indicated. For example, Cys-98 of TnC can be cross-linked using a photoreactive cross-linker (4-maleimidobenzophenone) to TnI in the region 103-110 (Leszyk *et al.*, 1987). Attachment of the photoaffinity probe (benzophenone moiety) to the α-amino group of TnI inhibitory peptide 104-115 and cross-linking to TnC results in labeling of the H-helix of the C-terminal domain of TnC (residues 154-159). Kobayashi *et al.* (1991) have shown that a mutant TnC containing a single Cys at residue 57 and modified with 4-maleimidobenzophenone can be cross-linked to TnI in the region 113-121. These results suggest that both the N- and C-terminal domains of TnC are in close proximity to the inhibitory region of TnI 104-115 and that TnC in the presence of TnI adopts a more compact conformation in solution (Wang *et al.*, 1987) than in the crystal structure of TnC (Sundaralingam *et al.*, 1985; Herzberg and James, 1985, 1988). The crystal structure shows two structurally independent domains linked by a long central helix (the N-terminal domain contains the low affinity Ca^{2+}-specific binding sites I and II and the C-terminal domain contains the high affinity Ca^{2+}-Mg^{2+} binding sites III and IV).

Wang *et al.* (1990) showed that binding of cations to the high affinity sites (sites III and IV) of a mutant TnC altered the environment around the amino acid at position 57 in the N-terminal domain (sites I an II). Grabarek *et al.* (1986) and Rosenfeld and Taylor (1985) showed that the binding of Ca^{2+} to the low affinity sites altered the environment around Cys 98 in C-terminal domain.

Lan *et al.* (1989) indicated that the preferred binding site for the TnI inhibitory peptide was the C-terminal domain of calmodulin (78-148) with an affinity comparable with that in intact calmodulin or TnC. However, there was binding to the N-terminal half of calmodulin (1-77) but the binding affinity was an order of magnitude less. Ip also inhibited the tryptic digest at the midpoint of the central helix linking the two domains of either TnC or calmodulin.

Swenson and Fredrickson (1992) have shown by intrinsic fluorescence that Ip containing a N-terminal Trp binds in a 1:1 stoichiometry to TnC and its fragments (N-terminal fragment, residues 1-97; C-terminal fragment, residues 98-159). Taken together the results of these authors and others (Van Eyk *et al.*, 1991; Leszyk *et al.*, 1988, 1990; Heidorn and Trewhella, 1988; Cheung and Wang, 1989) have suggested that the binding of TnI inhibitory peptide may be more complex than initial interaction studies suggested and may form a single binding site between the N- and C-terminal domains of TnC.

Perry *et al.* (1975) had proposed two mechanisms to describe how troponin C may interact with TnI to neutralize the inhibitory activity of TnI. First it was postulated that the two sites (referred to as Rp and Ip in this study) are located on the troponin I molecule so that interaction of one site with TnC takes place without physical obstruction of the other. In this case, the interaction of TnC at the N-terminal region of TnI in the presence of Ca^{2+}, leads to a conformational change that causes modification in the molecule in the region of Ip so that the Ip site is no longer available for interaction with actin. Though this scheme did not propose the strong interaction of TnC with the inhibitory peptide Ip in the presence of Ca^{2+}(Van Eyk & Hodges, 1988), this interaction is compatible with Perry's proposal. However, the large conformational changes in TnI were ruled out by studies showing that Ca^{2+} induced changes in the TnC/TnI complex were only slightly greater than the sum of those in the separate subunits as measured by circular dichroism (McCubbin *et al.*, 1973).

The second scheme requires that regions of troponin I (Rp and Ip) that interact with TnC and actin are located close together on the surface of the TnI molecule so that Ca^{2+}-induced interaction with TnC effectively prevents actin from interacting with the inhibitory region (Ip). Compatible with this scheme is that one of the chemical switches in muscle regulation involves the binding of Ip to actin preventing the S1-actin interaction (muscle relaxation). In the presence of Ca^{2+}, conformational changes in TnC result in the release of inhibition via TnC binding to the Ip region, thus allowing the interaction of S1 and actin (muscle contraction) (Syska *et al.*, 1976; Weeks and Perry, 1978; Grabarek *et al.*, 1981 and Grand et al., 1982). We have prepared monoclonal antibodies to Ip that bind to TnI and troponin in the presence or absence of Ca^{2+}, showing that the Ip region is exposed on the surface of troponin and available for interaction with either actin in the absence of Ca^{2+} or TnC in the presence of Ca^{2+}(Van Eyk *et al.*, personal communication).Since the binding of Ca^{2+} to TnC releases the inhibition caused by the TnI inhibitory region binding to actin-TM and it is well documented that the inhibitory region binds to TnC in the presence of Ca^{2+} (Syska *et al.*, 1976; Weeks and Perry, 1978; Grabarek *et al.*, 1981; Grand et al., 1982 and Van Eyk and Hodges, 1987), it was expected that the Ca^{2+}-dependent binding of Rp to TnC would assist TnC in releasing the inhibition of Ip. However, in this study the binding of Rp to TnC was shown to prevent the ability of TnC in the presence of Ca^{2+} to bind to the inhibitory region, Ip , and neutralize the inhibition of the acto-S1-TM ATPase activity caused by Ip. The Rp peptide seems to be a negative effector in this regulatory process.

The evidence that Rp can displace TnI from binding to TnC suggests that there is a subtle difference between Rp peptide binding to TnC and the Rp region of TnI binding to TnC. This result leads one to conclude that the presence of the remaining polypeptide chain of TnI regulates how the N-terminal region of TnI binds to TnC, which in turn, controls the conformational changes in TnC and exposes the binding sites on TnC for the inhibitory region of TnI in the presence of Ca^{2+}. The results of this study clearly support the conclusion that the Ca^{2+}-dependent regulatory process is more complex than previously thought. Although the binding of Ca^{2+} to TnC releases inhibition, there is a very subtle control process by the TnI region 1-40 which can modulate such release.

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CHAPTER V

CHARACTERIZATION OF THE BIOLOGICALLY IMPORTANT INTERACTION BETWEEN TROPONIN C AND THE N-TERMINAL REGION OF TROPONIN I

ABSTRACT

The N-terminal regulatory region of Troponin I, residues 1-40 (TnI1-40, regulatory peptide) has been shown to have a biologically important function in the interactions of troponin I and troponin C (Ngai and Hodges (1992) *J. Biol. Chem. 267,* 15715-15720). Truncated analogs corresponding to shorter versions of the N-terminal region (1-30, 1-28, 1-26) were synthesized by solid-phase methodology. Our results indicate that residues 1-30 of TnI comprises the minimum sequence to retain full biological activity as measured in the acto-S1-TM ATPase assay. Binding of the TnI N-terminal regulatory peptides [TnI1-30 and the N-terminal regulatory peptide (residues 1-40) labeled with the photoprobe benzoylbenzoyl group, BBRp] were studied by gel electrophoresis and photochemical cross-linking experiments under various conditions. Fluorescence titrations of TnI1-30 were carried out with TnC mutants that carry a single tryptophan fluorescence probe in either the N- or C-domain (F105W, F105W/C domain (88-162), F29W and F29W/N domain (1-90)) (Fig. V-1). Low Kd values ($Kd < 10^{-7}$ M) were obtained for the interaction of F105W and F105W/C domain (88-162) with TnI1-30. However, there was no observable change in fluorescence when the fluorescence probe was located at the N-domain of the TnC mutant (F29W and F29W/N domain (1-90)). These results show that the regulatory peptide binds strongly to the C-terminal domain of TnC.

INTRODUCTION

In our previous studies on the biological activity of the N-terminal region of TnI, we found that the TnI N-terminal peptides by themselves are not capable of interacting with the thin filament. However, when bound to TnC, the TnI N-terminal peptides could prevent the ability of TnC to neutralize the inhibition of the acto-S1-TM ATPase activity caused by either TnI or the TnI inhibitory peptide (Ip), residues 104-115 (Ngai and Hodges, 1992). In the present study, truncated TnI N-terminal peptides with deletion at the carboxyl terminus were studied (see Table V-1 for peptide sequences). The minimum sequence in the TnI N-terminal regulatory region that can retain biological function was identified as residues 1 to 30 and proven to be as described. Investigation on the binding of the TnI N-terminal peptide to TnC was performed using gel electrophoresis, photolysis and fluorescence studies. This work was carried out in collaboration with Drs. J. R. Pearlstone and L. B. Smillie.

RESULTS AND DISCUSSION

Effect of TnI N-terminal Peptides on the ability of TnC (+Ca^{2+}) to Release Ip (104-115) Inhibition of Acto-S1-TM ATPase — In agreement with our previous investigation (Ngai and Hodges, 1992), all TnI N-terminal peptides by themselves are not capable of interacting with the actin-TM filament and have no inhibitory effect on the acto-S1-TM ATPase activity. In Fig. V-2 (panel A), the acto-S1-TM ATPase activity was initially inhibited with TnI inhibitory peptide (Ip), followed by the release of inhibition by TnC in the presence of Ca^{2+} and in the absence or presence of TnI N-terminal peptides [TnI1-40, TnI1-30, TnI1-28 and TnI1-26]. In the presence of Ca^{2+}, TnC fully released the inhibition induced by Ip (from 26% to 100% ATPase activity). The interaction of the N-terminal TnI peptides with TnC (pre-incubation of TnC with the TnI N-terminal peptides) prevented TnC from fully releasing the acto-S1-TM ATPase inhibition induced by TnI inhibitory

Table V-1

Amino Acid Sequences of TnI Peptides [a]

TnI Inhibitory Peptide 104-115 (Ip)

104 115
Ac-GKFKRPPLRRVR-amide

TnI N-terminal Peptides

1 10 20 30 40
Ac-GDEEKRNRAITARRQHLKSVMLQIAATELEKEEGRREAEK-amide
(TnI 1-40)

1 10 20 30
Ac-GDEEKRNRAITARRQHLKSVMLQIAATELE-amide
(TnI 1-30)

1 10 20
Ac-GDEEKRNRAITARRQHLKSVMLQIAATE-amide
(TnI 1-28)

1 10 20
Ac-GDEEKRNRAITARRQHLKSVMLQIAA-amide
(TnI 1-26)

1 10 20 30 40
BB-GDEEKRNRAITARRQHLKSVMLQIAATELEKEEGRREAEK-amide[b]
BBRp (TnI 1-40)

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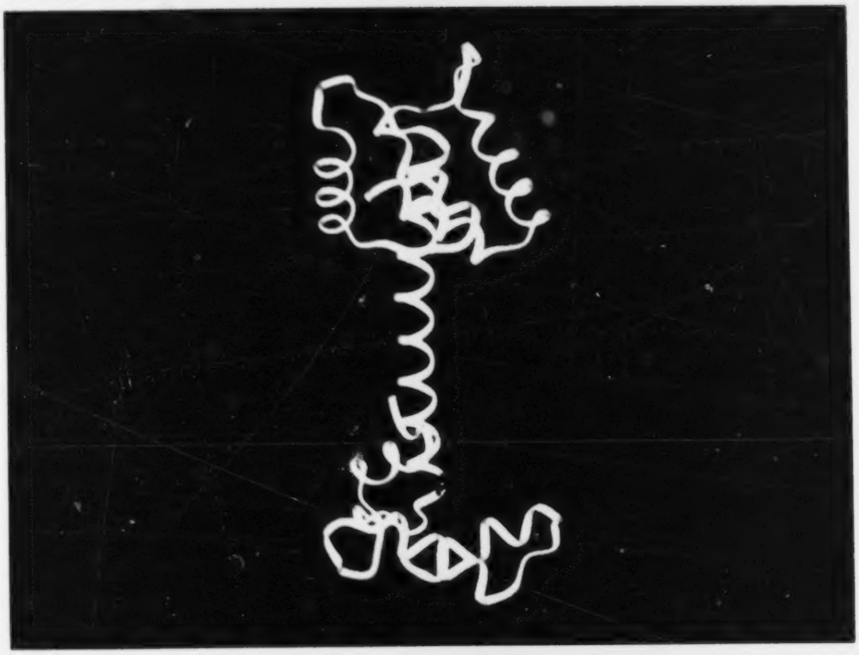
[a] These sequences are from primary sequence of rabbit skeletal troponin I (Wilkinson and Grand, 1975, 1978)

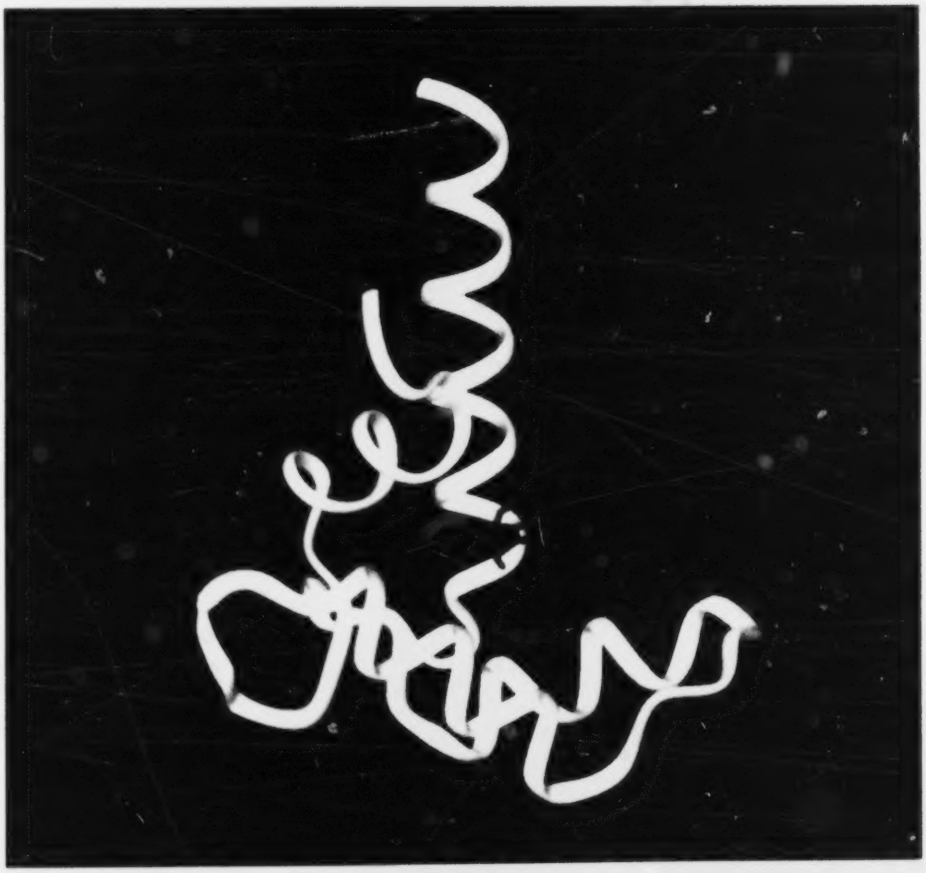
[b] The TnI regulatory peptide (TnI1-40, Rp) was synthesized with Gly-1 labeled $[\alpha\text{-}^{14}C]$ and covalently linked to a benzoylbenzoyl moiety (BB) to yield the BBRp peptide.

Fig. V-1. **TnC mutants (F105W, F105W/C domain (88-162), F29W and F29W/N domain (1-90))**. TnC mutant, polypeptide backbone in white ribbon and the corresponding trytophan probe is indicated in purple.

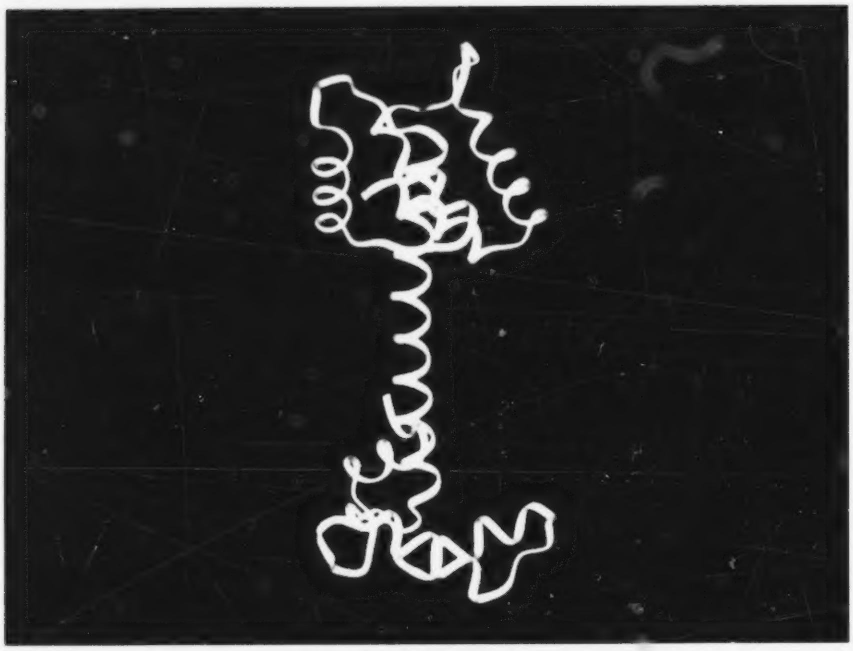
F105W



F105W/C domain



F29W



F29W/N domain

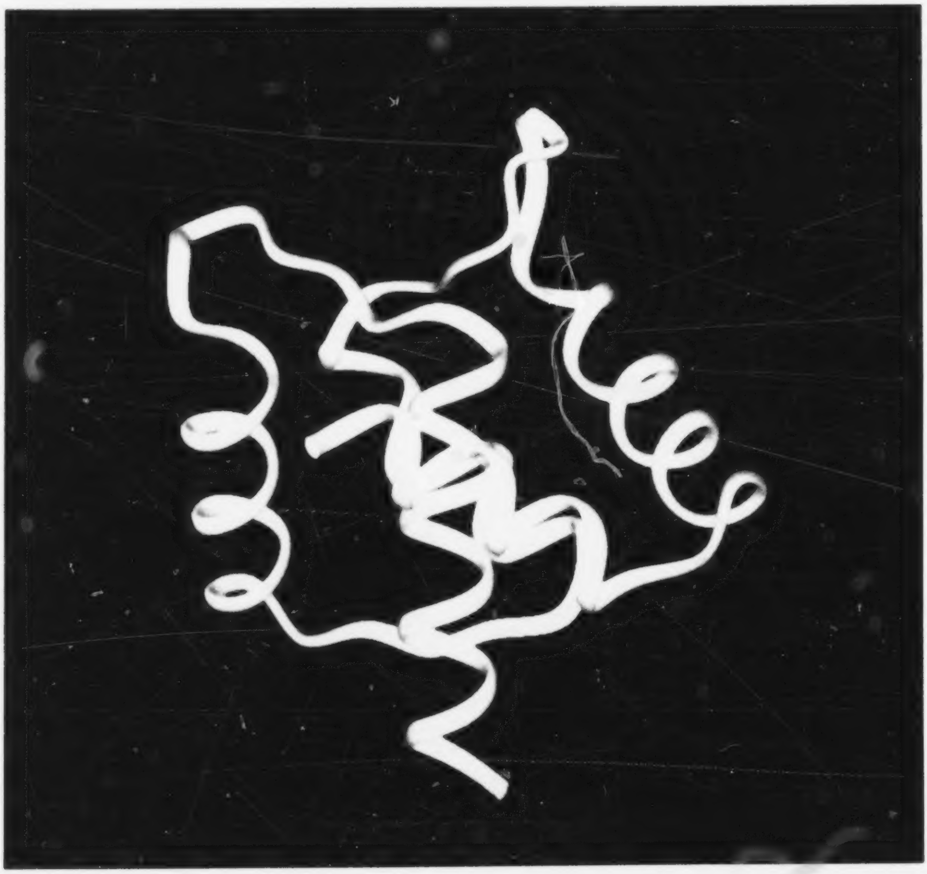
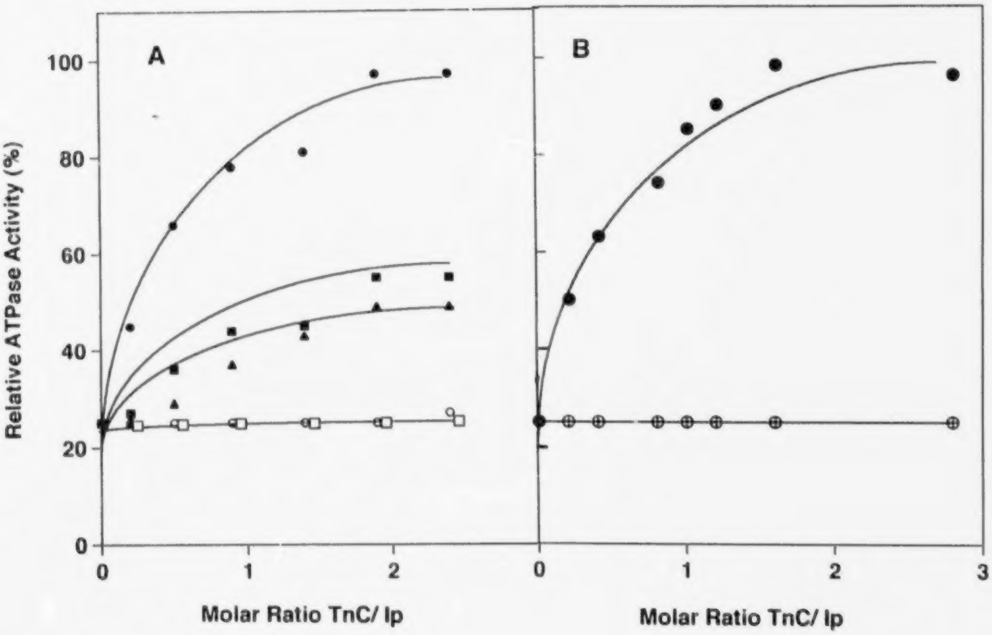


Fig. V-2. **Effect of the N-terminal TnI peptide on the ability of TnC (Ca^{2+}) to release the acto-S1-TM ATPase inhibition by TnI inhibitory peptide (Ip).** The S1, actin and TM concentrations were 3, 1.5 and 0.22 μM, respectively, giving a molar ratio of 14:7:1. Panel A: (●) TnC; (■) TnC + TnI1-26 [1:1.5]; (▲) TnC + TnI1-28 [1:1.5] (○) TnC + TnI1-30 [1:1.5]; (□) TnC + TnI1-40 [1:1.5]; Panel B: (○) TnC + TnI1-30 [1:1.5]; (+) TnC + BBRp [1:1.5]; (●) TnC.


A
B
Relative ATPase Activity (%)
0
20
40
60
80
100
0
1
2
0
1
2
3
Molar Ratio TnC/ Ip
Molar Ratio TnC/ Ip

peptide (Ip). Our present data demonstrated that TnI1-30 possesses the maximum ability in preventing TnC from releasing the Ip induced ATPase inhibition (identical biological activity with that of TnI1-40). Therefore, we conclude that TnI residues 1 to 30 comprise the minimum sequence for the biological activity of the TnI N-terminal region.

Effect of BBRp on the ability of TnC (+Ca^{2+}) to Release Ip (104-115) Inhibition off the Acto-S1-TM ATPase — TnI N-terminal regulatory peptide (residues 1-40) was synthesized with α-^{14}C-labeled Gly at position 1 and covalently linked benzoylbenzoyl (BB) moiety at the N-terminus to yield a photoactivatable radioactive peptide (BBRp). The biological activity of BBRp on the acto-S1-TM ATPase assay was studied (Fig. V-2, panel B). BBRp retains its ability in preventing TnC from neutralizing the Ip induced inhibition of the ATPase activity [identical activity with that of TnI1-30].

Interaction of TnC mutants with TnI1-30 — Analysis of the interaction between TnI1-30 peptide and TnC mutants [TnC (1-162), TnC C-domain (88-162) and TnC N-domain (1-90)] by native gel electrophoresis is shown in Fig. V-3 (gel A to C). Fig. V-3, gel A and B demonstrate that TnI1-30 is not capable of maintaining a stable complex with TnC mutants (intact TnC, TnC C-domain and TnC N-domain) in either metal free or Mg^{2+} buffer. Fig V-3, gel C, however indicates that TnI1-30 can result a stable complex with either intact TnC or TnC C-domain in the presence of Ca^{2+} and no complex formation is observed with TnC N-domain in the presence of Ca^{2+}. We concluded that the major binding site on TnC for the N-terminal regulatory region of TnI is located at the C-domain of TnC. Our results are consistent with a previous observation that TnI residues 1-116 (TnI$_{1-116}$) and residues 1-98 (TnI$_{1-98}$) are interacting predominantly with TnC C-domain (Farah *et al.*, 1994).

Complex Formation between TnC and TnI N-terminal Peptide Before and After Photolysis — Fig. V-3, gel D, lanes 2 and 3 show that both TnI1-30 and BBRp are capable of forming a stable non-covalent complex with TnC in the presence of Ca^{2+}. However, photolysis of a mixture of the native rabbit skeletal TnC and BBRp (1:2 molar

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Fig. V-3. **Polyacrylamide gel electrophoresis**. The native 10 % polyacrylamide gel was polymerized in a Tris/glycine buffer, pH 8.6 and DTT (1 mM) at i) 1 mM EGTA (metal free buffer), ii) 1 mM EGTA and 5 mM $MgCl_2$ (Mg^{2+} buffer) or iii) 5 mM $CaCl_2$ Ca^{2+*}.

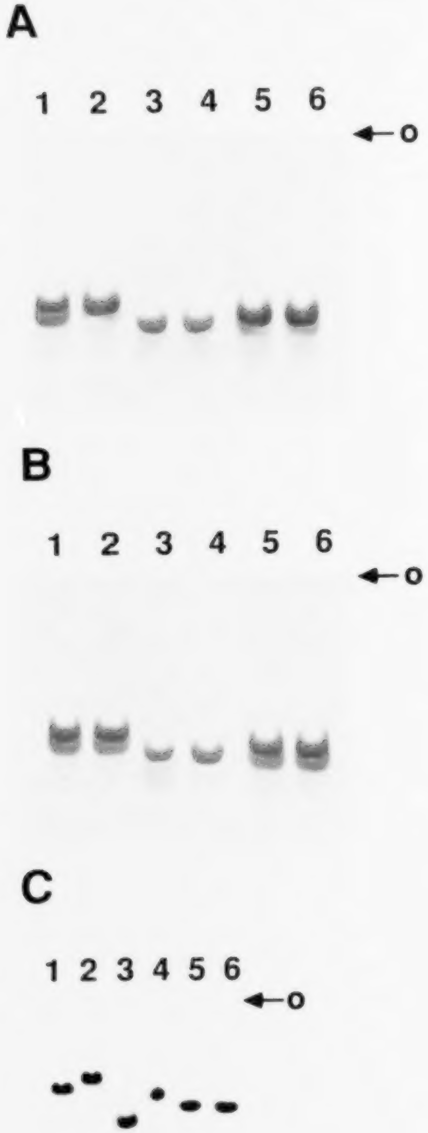
A. *TnC/TnI1-30, TnC C-domain/TnI1-30 and TnC N-domain/TnI1-30 Interaction in metal free buffer*: Lane 1, TnC (1 nmole); Lane 2, TnI1-30 (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 3, TnC C-domain (2 nmole); Lane 4, TnI1-30 (3 nmole) and TnC C-domain (2 nmole) equilibrated for 1 hr; Lane 5, TnC N-domain (2 nmole); Lane 6, TnI1-30 (3 nmole) and TnC N-domain (2 nmole) equilibrated for 1 hr.

B. *TnC/TnI1-30, TnC C-domain/TnI1-30 and TnC N-domain/TnI1-30 Interaction in Mg^{2+} buffer*: Lane 1, TnC (1 nmole); Lane 2, TnI1-30 (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 3, TnC C-domain (2 nmole); Lane 4, TnI1-30 (3 nmole) and TnC C-domain (2 nmole) equilibrated for 1 hr; Lane 5, TnC N-domain (2 nmole); Lane 6, TnI1-30 (3 nmole) and TnC N-domain (2 nmole) equilibrated for 1 hr.

C. *TnC/TnI1-30, TnC C-domain/TnI1-30 and TnC N-domain/TnI1-30 Interaction in Ca^{2+} buffer*: Lane 1, TnC (1 nmole); Lane 2, TnI1-30 (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 3, TnC C-domain (2 nmole); Lane 4, TnI1-30 (3 nmole) and TnC C-domain (2 nmole) equilibrated for 1 hr; Lane 5, TnC N-domain (2 nmole); Lane 6, TnI1-30 (3 nmole) and TnC N-domain (2 nmole) equilibrated for 1 hr.

D. *Photolysis Experiment* : Lane 2 and lane 3 correspond to the study of complex formation before photolysis in the presence of Ca^{2+}. Lane 5 to 7 correspond to the study of covalently crosslinked TnC/BBRp complex after photolysis. Lanes 1, 4 and 8 are TnC controls (1 nmole). Lane 2 shows the non-covalent TnC/TnI1-30 complex formed by mixing TnI1-30 (0.75 nmole) and TnC (0.5 nmole) after equilibration for 1 hr. Lane 3 shows the non-covalent TnC/BBRp complex formed by mixing BBRp (1.5 nmole) and TnC (1 nmole) after equilibration for 1 hr. Lane 5, 6 and 7 show TnC/BBRp covalently linked complex (1 nmole) formed in metal free buffer, in Mg^{2+} buffer, and in Ca^{2+} buffer, respectively.

* TnI peptides do not enter gel.


A
1 2 3 4 5 6
o
B
1 2 3 4 5 6
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C
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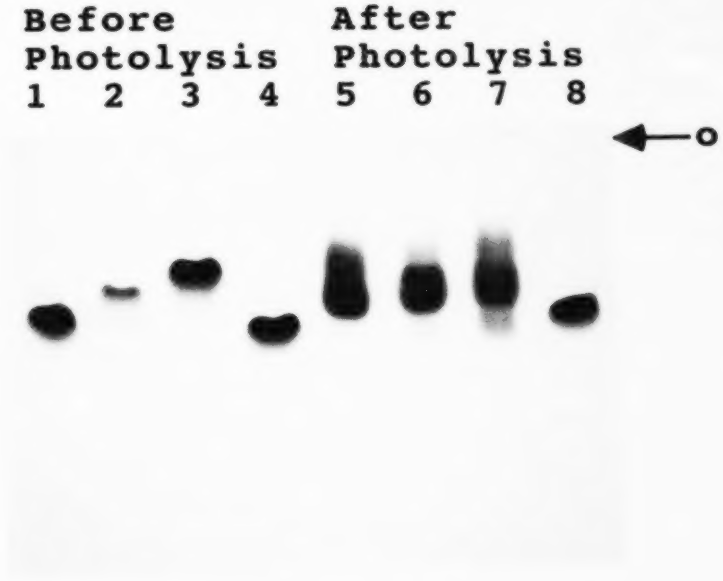
D


Before Photolysis
After Photolysis
1 2 3 4 5 6 7 8
o

ratio) in either metal free, Mg^{2+} or Ca^{2+} buffers yielded a covalent TnC/BBRp complex (Fig. V-3, gel D; lanes 5, 6 and 7 respectively). Analysis of the complex by native gel electrophoresis indicated that photochemical crosslinking between TnC and the BBRp peptide was complete and that the complex isolated by reversed-phase HPLC consisted of TnC and BBRp in a 1:1 molar ratio when measured by amino acid analysis and radioactivity. The finding that TnC/BBRp covalent complex could be formed with all states of TnC (apo, Mg^{2+} and Ca^{2+}) indicates the regulatory region of TnI (residues 1-40) is always capable of interacting with TnC under the above buffering conditions (strongest interaction in the presence of Ca^{2+}) and the N-terminus of BBRp is in close enough proximity to allow for crosslinking to occur during photolysis.

Impact of TnI1-30 on the fluorescence of TnC mutants [F105W, F105W/C domain (88-162), F29W and F29W/N domain (1-90)] — There is no significant fluorescence change observed upon binding of TnI1-30 to the two TnC F29W mutants [F29W and F29W/N domain (1-90)] which carry a single fluorescence probe at residue-29 of the N-terminal domain of TnC. Emission fluorescence spectra of F105W and F105W/C domain (88-162) are shown in Fig. V-4. Both spectra of the TnC mutants have a common λmax at 335 nm. However, upon binding of TnI1-30 onto the corresponding TnC mutant, there is a blue shift in the spectra of the complex formed and a common λmax at 330 nm for both F105W and F105W/C domain (88-162) (Fig. V-4). This indicates that the local environment of Trp-105 in either F105W or F105W/C domain (88-162) is altered upon the binding of TnI1-30.

Fluorescence titration experiments of the TnC mutants [F105W and F105W/C domain (88-162)] with TnI1-30 — The addition of TnI1-30 to the TnC mutants resulted a series of difference fluorescence emission spectra which allow us to determine the binding constant of TnI1-30 to the corresponding TnC mutant. The binding curves for both TnC mutants are shown in Fig. V-5 which demonstrated that TnI1-30 binds more tightly to F105W than F105W/C domain (88-162). The titration was complete at about a 1 to 1 molar

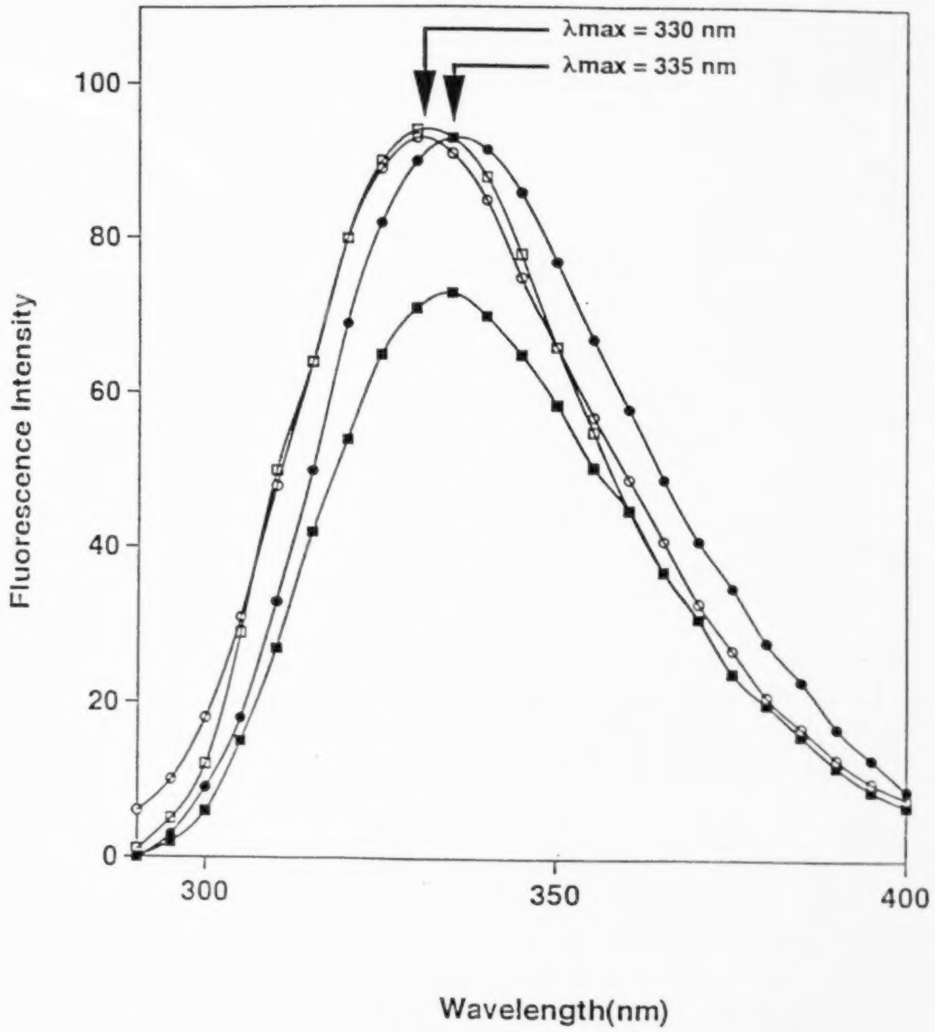


Fig. V-4. **Fluorescence Spectra.** Emission fluorescence spectra : (●) F105W; (○) F105W + TnI1-30; (■) F105W/C domain (88-162) and (□) F105W/C domain (88-162) + TnI1-30. λmax for the spectra of both F105W and F105W/C domain (88-162) is at 335 nm. λmax for the spectra of both F105W + TnI1-30 and F105W/C domain (88-162) + TnI1-30 is at 330 nm as indicated in the figure. Concentration for F105W is 4.6 μM and that of F105W/C domain is 4.25 μM. Buffer conditions are described in Materials and Methods section.

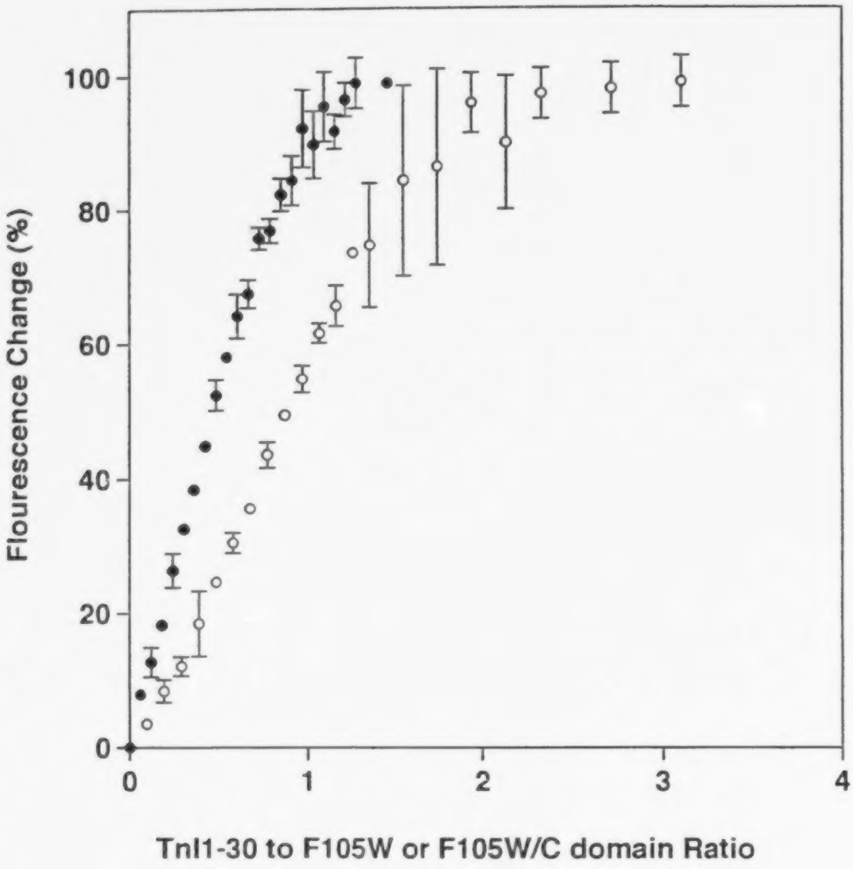


Fig. V-5. **Fluorescence Titration.** Experiments monitoring Trp-105 emission at 330 nm with stepwise addition of TnI(1-30). The average data points are shown (●) F105W and (○) F105W/C domain (88-162), with the range of values indicated by bars.

ratio of TnI1-30 to F105W. The evaluation of the binding constant is based on the application of the following modified equation (Berger and Levit, 1973) which is based on the mass action law. Linearization of the equation assists in obtaining the parameters Co and Kd graphically.

$$Rp\left(\frac{Fmax}{F} - 1\right) = Co\left(1 - \frac{F}{Fmax}\right) + Kd$$

where Rp is the concentration of TnI1-30 added (μM, 10^{-6}M), F is the corresponding observed fluorescence, Fmax is the maximum fluorescence change observed, Co (slope term in Fig. V-6) is the protein concentration (μM, 10^{-6}M), Kd (the y-intercept in Fig. V-6) is the dissociation constant (μM, 10^{-6}M). Kd values for both of the F105W and F105W/C domain (88-162) towards the interaction of TnI1-30 are found to be less than 10^{-7} M^{-1}.

Studies on the binding behavior of the TnI1-30 with TnC in either the presence or absence of Ca^{2+} allows us to understand the Ca^{2+}-dependent TnI/TnC interaction within the troponin complex. By knowing this, we can understand more about the influence of divalent cations on the TnI/TnC interaction. For this purpose, the troponin I N-terminal regulatory region (1-40) was synthesized with Gly-1 labeled [α-^{14}C] and a covalently linked benzoylbenzoyl (BB-) moiety at the N-terminus to yield the photoactivatable radioactive peptide (BBRp) to determine the feasibility of crosslinking it to rabbit skeletal TnC. Results of the photochemical crosslinking experiment demonstrated that BBRp was able to crosslink to TnC molecule in its apo, Ca^{2+} or Mg^{2+} states. In other words, the TnI N-terminal regulatory sequence (residues 1-40) is always capable of interacting with TnC regardless of the metal ion state of TnC. This is consistent with our previous ATPase results which show that the TnI N-terminal regulatory peptides can prevent the release of Ip inhibition by TnC in both Ca^{2+} and Mg^{2+} ATPase buffer (Ngai and Hodges, 1992).

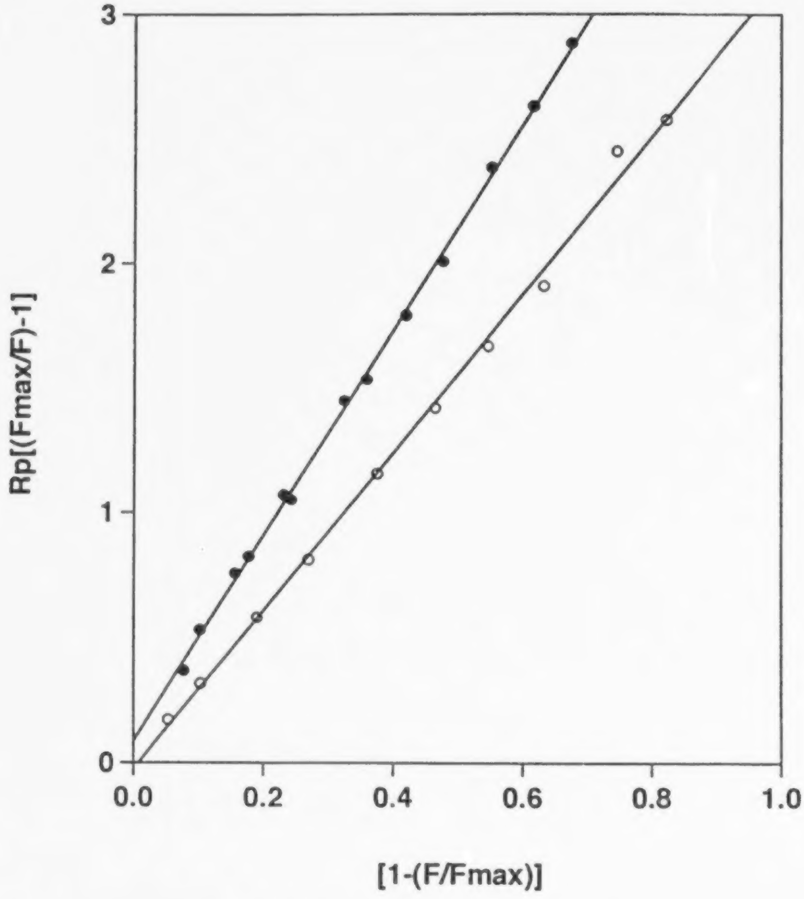


Fig. V-6. **Determination of the Binding Constant for F105W and F105W/C domain (88-162).** (●) F105W; (○) F105W/C domain (88-162). Ka for both intact F105W and F105W/C domain (88-162) are greater than 10^7 M^{-1}

Farah *et al.* (1994) demonstrated that a TnI mutant (TnI$_{103-182}$) which has in its primary sequence the inhibitory region of TnI (residues 104-115) extended to the C-terminus (residues 116-182) is capable of regulating the acto-S1-TM ATPase activity in a calcium dependent manner. Another two TnI mutants, TnI$_{1-98}$ which carries the N-terminal domain of TnI (residues 1-98) and TnI$_{1-116}$ (residues 1-116) are both capable of interacting with TnC in either the presence or absence of Ca^{2+}. However, stable TnC/TnI mutant complex formation with either whole TnC or TnC C-domain can only be detected with TnI mutants that carry the N-terminal region (residues 1-98). Our present result indicates a similar observation that TnI1-30 can form a stable complex with both TnC or C-domain in the presence of Ca^{2+} and no complex formation was observed with TnC N-domain.

Sheng *et al* (1992) expressed a TnI recombinant deletion mutant (TnId57), which lacks residues 1-57, in a bacterial expression system. This TnI mutant inhibited acto-S1-ATPase activity and such inhibition could be fully neutralized by TnC in the presence of Ca^{2+} (100% ATPase activity) and partially neutralized by TnC in the absence of Ca^{2+} (50% ATPase activity). Affinity chromatographic studies of this TnI mutant TnId57 indicates that TnId57 appears to lack the region of TnI required for Ca^{2+} -Mg^{2+} site-dependent interaction and has a weakened metal independent interaction.

The above studies have demonstrated that the N-terminal region of TnI interacts mainly with the C-domain of TnC (the Ca^{2+}/Mg^{2+} sites III and IV of TnC) and they both concluded that the N-terminal region of TnI plays a structural rather than a functional role in the Ca^{2+} sensitive control of the actomyosin ATPase. However, they both fail to explain the ability of the N-terminal TnI peptides (TnI1-30 and TnI1-40) which when bound to TnC prevents the ability of TnC to release the inhibition of the acto-S1-TM ATPase activity caused by the inhibitory region of TnI. Our native PAGE gel studies indicate once again TnI N-terminal regulatory region (residues 1-40 or residues 1-30) are capable of forming a very tight complex with TnC in the presence of Ca^{2+}. The displacement of Ip from the hydrophobic groove of TnC C-domain upon addition of TnI1-30 or TnI1-40 reveals that the TnI N-terminal region may be a negative effector of the regulatory process. Wang *et al.* (1990) demonstrated that binding of cations to the high affinity sites (sites III and IV) of a mutant TnC altered the environment around the amino acid at position 57 in the N-terminal domain (sites I and II). Grabarek *et al.* (1986) and Rosenfeld and Taylor (1985) showed that the binding of Ca^{2+} to the low affinity sites altered the environment around Cys 98 in the C-terminal domain. These findings indicated that the N and C-terminal domains of TnC are communicating with each other upon the binding of Ca^{2+} or Mg^{2+}. Therefore, there must be a modulating event which governs the release of the TnI inhibitory region (Ip) region from TnC by the N-terminal region of TnI. Although the N-domain of TnC contains the Ca^{2+}- regulatory sites that control muscle contraction, the two important regions of TnI (Ip, residues 104-115 and TnI1-30, residues 1-30) bind to the C-domain. This raises the question of how Ca^{2+}- binding to the N-domain of TnC transmits this information to the C-domain of TnC.

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Wang, Z., Sarkar, S., Gergely, J., and Tao, T. (1990) *J. Biol. Chem.* **265**, 4953-4957.

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CHAPTER VI

STRUCTURAL AND FUNCTIONAL STUDIES ON TROPONIN I AND TROPONIN C INTERACTIONS

ABSTRACT

Troponin I (TnI) peptides (TnI inhibitory peptide residues 104-115, Ip; TnI regulatory peptide resides 1-30, TnI1-30), recombinant Troponin C (TnC) and Troponin I mutants were used to study the structural and functional relationship between TnI and TnC. Our results reveal that an intact central D/E helix in TnC is required to maintain the ability of TnC to release the TnI inhibition of the acto-S1-TM ATPase activity. Ca^{2+}-titration of the TnC-TnI1-30 complex was monitored by circular dichroism. The results show that binding of TnI1-30 to TnC caused a three-folded increase in Ca^{2+} affinity in the high affinity sites (III and IV) of TnC. Gel electrophoresis and high performance liquid chromatography studies show that the sequences of the N-terminal and C-terminal regions of TnI interact in an anti-parellel fashion with the corresponding N- and C-domain of TnC. Our results indicate that the N- and C-terminal domains of TnI which flank the TnI inhibitory region (residues 104 to 115) also play a vital role in modulating the Ca^{2+}- sensitive release of the TnI inhibitory region by TnC within the muscle filament. A modified schematic diagram of the TnC/TnI interaction is proposed.

INTRODUCTION

Our laboratory has demonstrated that TnI residues 104-115 (Ip) comprise the minimum sequence necessary for the inhibition of actomyosin ATPase activity (Talbot and Hodges, 1979, 1981a,b). It has been concluded that the Ca^{2+}- dependent switch between muscle relaxation and contraction involves a switching event of the TnI inhibitory region (residues 104-115) between actin-TM and TnC, respectively (Syska *et al.*, 1976; Weeks and Perry, 1978; Talbot and Hodges, 1979, 1981a, 1981b; Grabarek *et al.*, 1981; Grand et al., 1982; Katayama and Nozaki, 1982; Cachia *et al.*, 1983, 1986; Van Eyk and Hodges, 1987, 1988). A computer generated three-dimensional model demonstrating the interaction between TnC C domain and the TnI inhibitory region (peptide) was proposed (Ngai *et al.*, 1994). Although this inhibitory site of TnI is of major importance, it is not the only Ca^{2+}-sensitive TnI/TnC binding site (Syska *et al.*, 1976; Weeks and Perry, 1978; Grabarek *et al.*, 1981 and Ngai and Hodges 1992). We have demonstrated an important interaction between the N-terminal region of TnI and TnC and indicated that the N-terminal domain of TnI is not just playing a structural role for anchoring troponin complex in the thin filament via TnT (Ngai and Hodges, 1992). Characterization of this interaction between TnC and the TnI N-terminal region was carried out in Chapter V.

In the present chapter, TnI peptides (See Table VI-1 for peptides used in this study) and recombinant mutants of TnC and TnI were used to investigate the Ca^{2+}- sensitive TnI/TnC interaction that governs the events of muscle contraction and relaxation. This chapter summarizes our investigations on the TnI/TnC interactions. This research was carried out in collaboration with Drs. J. R. Pearlstone, L. B. Smillie, C. S. Farah and F. C. Reinach.

RESULTS

Effect of TnI1-30 on recombinant TnC mutants in release of the Acto-S1-TM ATPase Inhibition by TnI Inhibitory Peptide (Ip) — The acto-S1-TM ATPase activity was inhibited with TnI inhibitory peptide, residues 104-115 (Ip), followed by the release of inhibition by TnC mutant in the presence of Ca^{2+} and in the absence or presence of TnI N-terminal peptide, TnI1-30 (Fig. VI-1). In the presence of Ca^{2+}, the intact wild type chicken TnC fully released the inhibition induced by Ip (from 28% to 90% ATPase activity) in a manner similar to that of rabbit skeletal TnC (not shown). The recombinant chicken TnC C-domain only partially releases the Ip inhibition (from 28% to about 50%

Table VI-1

Amino Acid Sequences of TnI Peptides [a]

TnI Inhibitory Peptide 104-115 (Ip)

104 115
Ac-GKFKRPPLRRVR-amide

TnI N-terminal Peptides

1 10 20 30 40
Ac-GDEEKRNRAITARRQHLKSVMLQIAATELEKEEGRREAEK-amide
(TnI1-40)

1 10 20 30
Ac-GDEEKRNRAITARRQHLKSVMLQIAATELE-amide
(TnI1-30)

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[a] These sequences are from primary sequence of rabbit skeletal troponin I (Wilkinson and Grand, 1975, 1978)

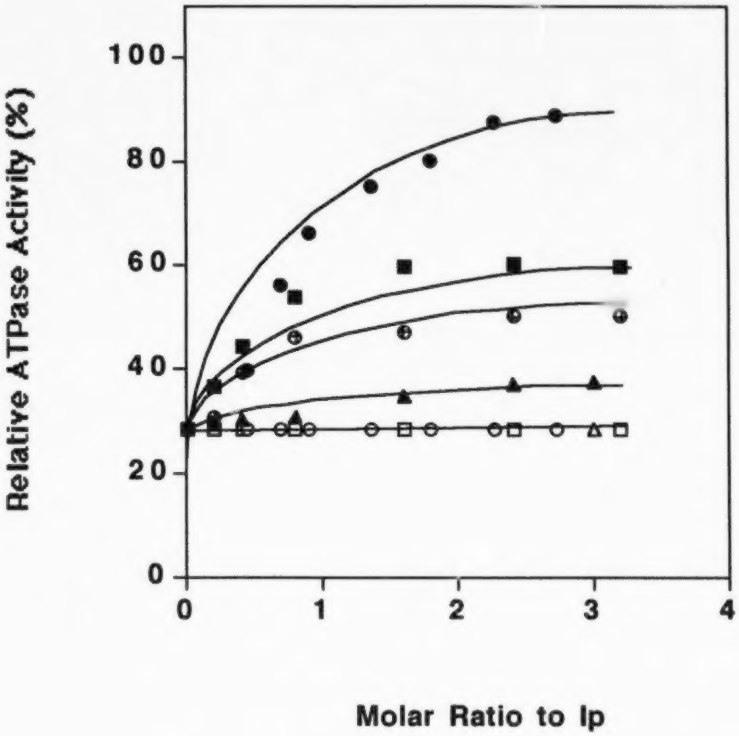

Fig. VI-1. **Effect of N-terminal TnI peptide on TnC mutant (Ca^{2+}) in release of acto-S1-TM ATPase inhibition by TnI inhibitory peptide (Ip).** The S1, actin and TM concentrations were 3, 1.5 and 0.22 μM, respectively, giving a molar ratio of 14:7:1. (●) TnC; (■) TnC C-domain; (▲) TnC N-domain; (⊕) TnC C-domain and TnC N-domain at a 1:1 molar ratio; (○) TnC + TnI1-30; (□) TnC C-domain + TnI1-30; (Δ) TnC N-domain + TnI1-30.

ATPase activity) whereas, the recombinant TnC N-domain was poorly effective in releasing the Ip inhibition (from 28% to 38% ATPase activity). The binding of TnI1-30 to TnC and TnC C-domain prevented neutralization of the Ip inhibition. Interestingly, there was only a partial release of the Ip induced inhibition (from 28% to 43% ATPase activity) by a molar equivalent mixture of C- and N-domains (pre-incubated at 1 to 1 molar ratio of C- and N-domains for 1 hour before the titration); the result is similar to that of the C-domain (Fig. VI-1). These results indicate intact D/E helix which links the two domains of TnC is essential in providing the optimum interacting interface for Ip for full biological function of the TnC molecule. In addition, only intact chicken TnC is capable of partially neutralizing the Ip induced inhibition in the absence of Ca^{2+} (results not shown).

Study of the interaction between TnI1-30 and TnC mutants by size-exclusion (SEC) and Reversed-Phase liquid chromatography (RPC) — The interactions between TnI1-30 and recombinant TnC mutants in the presence of Ca^{2+} were further studied using HPLC methodology. Fig. VI-2, panel A (left) shows the SEC run of the pre-formed TnC/TnI1-30 complex. The complex peak was collected from the size-exclusion run followed by reversed-phase chromatography. TnC and TnI1-30 were easily separated on RPC (panel A, right). The complex (TnC/TnI1-30) was shown by RPC to consist of a 1 : 1 ratio of TnC and TnI1-30 (integration of peak areas). These results indicate that the shorter version of TnI N-terminal peptide, TnI1-30 (residues 1 to 30) was still capable of maintaining a stable complex with TnC ($+Ca^{2+}$) during SEC and this is in agreement with our previous investigation using larger TnI N-terminal regulatory peptide, residues 1-40 (Ngai and Hodges, 1992). Panel B (left) shows the SEC run of the pre-formed TnC C-domain-TnI1-30 complex. The complex peak was collected from the size-exclusion run followed by reversed-phase chromatography (panel B, right). The TnC C-domain/TnI1-30 complex was shown by RPC to consist of a 1 : 1.5 ratio of TnC and TnI1-30 respectively (integration of peak areas). Although these results demonstrated the ability of TnI1-30 in

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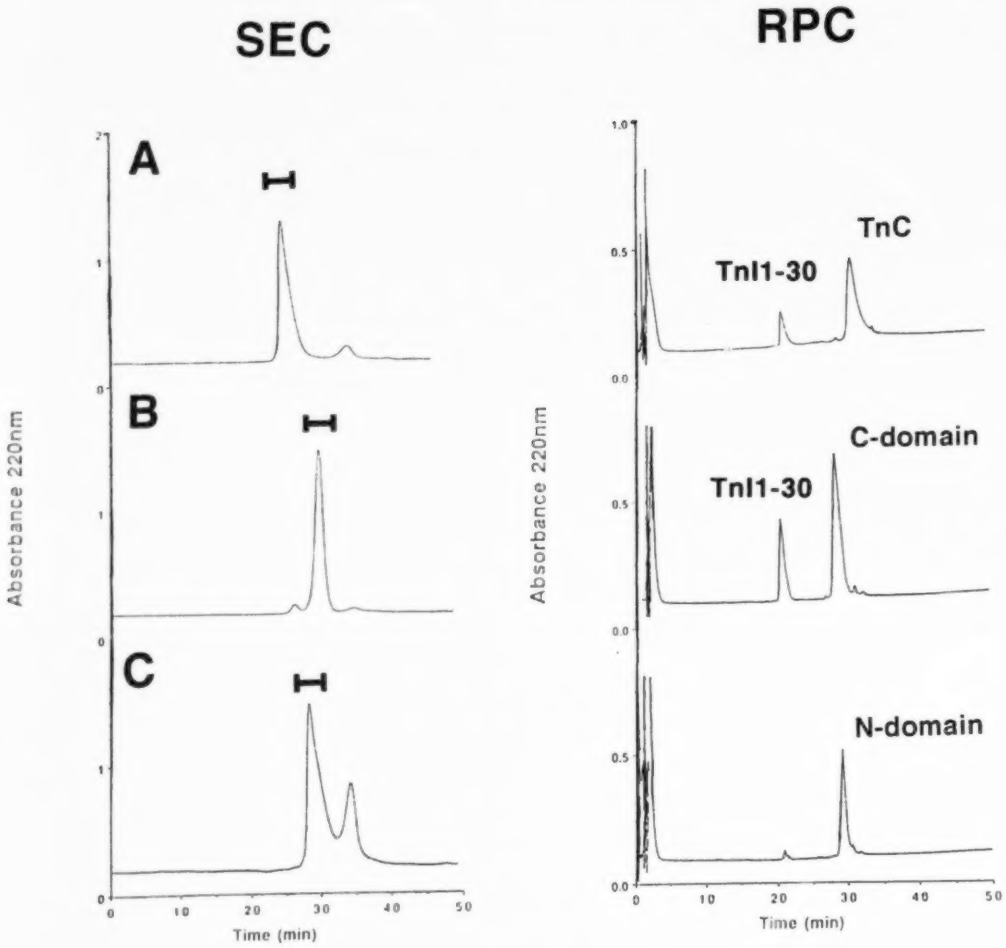
Fig. VI-2. **Study of the interaction between TnI1-30 and TnC mutant by HPLC.** Panel A (left), TnC (8 nmoles in 100 μl) and TnI1-30 (16 nmoles in 80 μl) were pre-incubated together (1:2 TnC/TnI1-30 molar ratio) for 1 h to form a TnC/TnI1-30 complex in the presence of Ca^{2+}. The pre-incubated mixture (150 μl) was loaded onto the SEC column and the complex (indicated by the horizontal bar on the left panel) was collected and loaded onto the microbore reversed-phase column (panel A, right). Panel B (left), TnC C-domain (16 nmoles in 100 μl) and TnI1-30 (32 nmoles in 160 μl) were pre-incubated together (1:2 C-domain/TnI1-30 molar ratio) for 1 h to form a TnC C-domain/TnI1-30 complex in the presence of Ca^{2+}. The pre-incubated mixture (220 μl) was loaded onto the SEC column and the complex (indicated by the horizontal bar on the left panel) was collected and loaded onto the microbore reversed-phase column (panel B, right). Panel C (left), TnC N-domain (32 nmoles in 200 μl) and TnI1-30 (64 nmoles in 320 μl) were pre-incubated together (1:2 N-domain/TnI1-30 molar ratio) for 1 h in the presence of Ca^{2+}. The pre-incubated mixture (500 μl) was loaded onto the SEC column and the peak of interest (indicated by the horizontal bar on the left panel) was collected and loaded onto the microbore reversed-phase column (panel A, right). See Materials and Methods section for conditions of incubation and running buffers for the SEC and RPC runs.


SEC
RPC
A
B
C
Absorbance 220nm
Absorbance 220nm
TnC
TnI1-30
C-domain
TnI1-30
N-domain
0
1
2
0.0
0.5
1.0
0
10
20
30
40
50
Time (min)
Time (min)

maintaining a stable complex with TnC C-domain (+Ca^{2+}) during SEC, the presence of a stable complex of TnI1-30 with TnC N-domain could not be demonstrated (see panel C, left and right). In addition, there is no stable complex formation by SEC for both intact TnC and TnC C-domain in the absence of Ca^{2+} . These observations indicate a significant reduced binding affinity between TnI1-30 and TnC or TnC C-domain in the absence of Ca^{2+}. This demonstrates the Ca^{2+}-sensitive nature of complex formation between TnC and TnI N-terminal peptides.

Ca^{2+} Titration Profile of Rabbit Skeletal TnC and the TnC/TnI1-30 Complex as monitored by far-UV Circular dichroism — The far UV CD calcium titration curves of rabbit skeletal TnC and the TnC/TnI1-30 complex are shown in Fig. VI-3. The apparent binding constants for Ca^{2+} of the low and high affinity sites of TnC are shown in Table VI-2. The pKd_1 values for the low affinity sites (sites I and II) of TnC and TnC/TnI1-30 complex are 5.408 and 5.519 respectively. However, for that portion of the titration curve attributable to the high affinity sites, the structural transition of the TnC/TnI1-30 complex was shifted to higher pCa value with a pKd_2 value of 7.617 as compared with a value of 7.154 for the rabbit skeletal muscle TnC protein. This shift in $-\log K_1$ values demonstrates an increase in Ca^{2+} affinity (three-fold) of sites III and IV of the TnC/TnI1-30 complex (Ka values of 1.4×10^7 M^{-1} and 4.2×10^7 M^{-1} for TnC and TnC/TnI1-30 complex, respectively).

Effect of TnI1-30 on the Interaction between Recombinant TnC and TnI mutants — Complex Formation studies - Farah *et al.* (1994) demonstrated that the N-terminal region of TnI interacts with C-domain of TnC and C-terminal region of TnI interacts with N-domain of TnC. Results from Fig. VI-4a is in agreement with their results and we have incorporated the TnI N-terminal regulatory peptide (TnI1-30) into the binding studies.

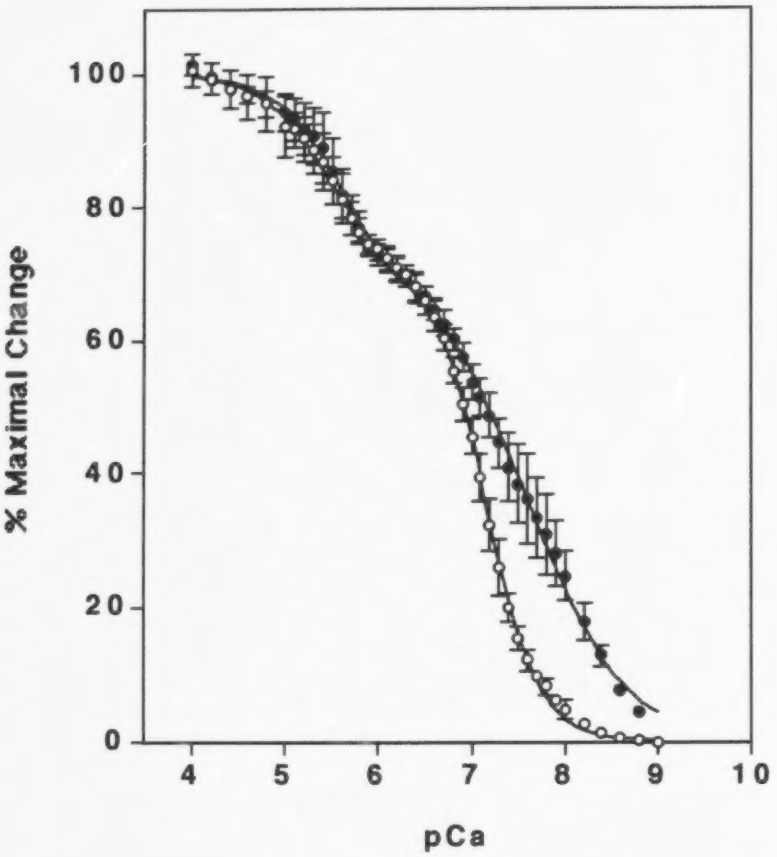


Fig. VI-3. **Effect of TnI regulatory peptide, TnI1-30 on Ca^{2+} binding to rabbit skeletal muscle TnC monitored by circular dichroism.** The percent maximal change in ellipticity of TnC in the presence (●) and absence (○) of TnI1-30 is plotted versus pCa value. A 1:1 molar equivalent of TnI1-30 and TnC was used and the curves were calculated by a computer program that best fits the experimental data to a curve composed of two binding constants. Error bars indicate average standard deviations resulted from six titrations. See method section for experimental conditions.

Table VI-2

Effect of TnI Regulatory Peptide (TnI1-30) on the pKd Values and Hill Coefficients for the Low- and High-Affinity Ca^{2+} Binding Sites of the Skeletal TnC

	Low-affinity site	High-affinity site
	pKd_1	pKd_2
TnC	5.408	7.154
TnC/TnI1-30	5.519	7.617

Fig. VI-4a (panel A) indicates that all TnI truncated analogs (TnI, TnI$_{103-182}$, TnI$_{1-116}$ and TnI1-30) are capable of forming a stable complex with TnC on the native gel (+Ca^{2+}). However, TnI does not bind as tightly as the other TnI truncated analogs which lack either the C or N-terminal region (TnI$_{1-116}$, TnI$_{103-182}$) or carries only the N-terminal regulatory sequence, residues 1-30 (TnI1-30). All TnI analogs (TnI, TnI$_{103-182}$, TnI$_{1-116}$ and TnI1-30) are capable of interacting with the C-domain in the presence of Ca^{2+} (panel B of Fig. VI-4a). Interaction between TnI$_{103-182}$ and C-domain does not result in a discrete band on the native gel. Importantly, only TnI and TnI$_{103-182}$ can form stable complexes with the N-domain of TnC (+Ca^{2+}) (Fig. VI-4a, panel C).

Competition Studies - As shown in Fig. VI-4b (panel A, left and right), TnI1-30 can displace TnI from either the TnC/TnI complex or TnC C-domain/TnI complex (lane 4 on both gels). Since native TnI molecule carries the same sequence of TnI1-30 in its primary structure, it suggests that the presence of TnI region other than residues 1-30 in the native TnI molecule reduces the strength of the TnC/TnI interaction. Fig. VI-4b (panel B, left) shows that TnC/TnI$_{103-182}$ interaction is tighter than TnC/TnI1-30 interaction for the fact that TnI1-30 is no longer able to displace TnI$_{103-182}$ from the TnC/TnI$_{103-182}$ complex (lane 4). The absence of the TnI region spanning residues 1 to 102 results in a tight interaction between TnI$_{103-182}$ and TnC, in other words, the presence of residues 1-102 in TnI reduces the strength of TnI/TnC interaction. However, consider that there is no overlapping sequence found between TnI$_{103-182}$ and TnI1-30; TnI$_{103-182}$ interacts predominantly with the TnC N-domain (Fig. VI-4a, panel C) (Farah *et al.*, 1994) whereas TnI1-30 interacts predominantly with the C-domain of TnC. It may suggest the formation of a ternary complex consists of TnC, TnI1-30 and TnI$_{103-182}$. However, it is also possible that TnI$_{103-182}$ when bound to TnC N-domain results in weakening the interaction between TnI1-30 and the C-domain of TnC. C-domain-TnI1-30 complex is not affected with the introduction of TnI$_{103-182}$ (lane 4 of Fig. VI-4b (panel B, right)). In

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Fig. VI-4. **Polyacrylamide gel electrophoresis.** The native polyacrylamide gel (10% crosslinked) was polymerized in a Tris/glycine buffer, pH 8.6 with Ca^{2+} (5 mM) and DTT (1 mM).

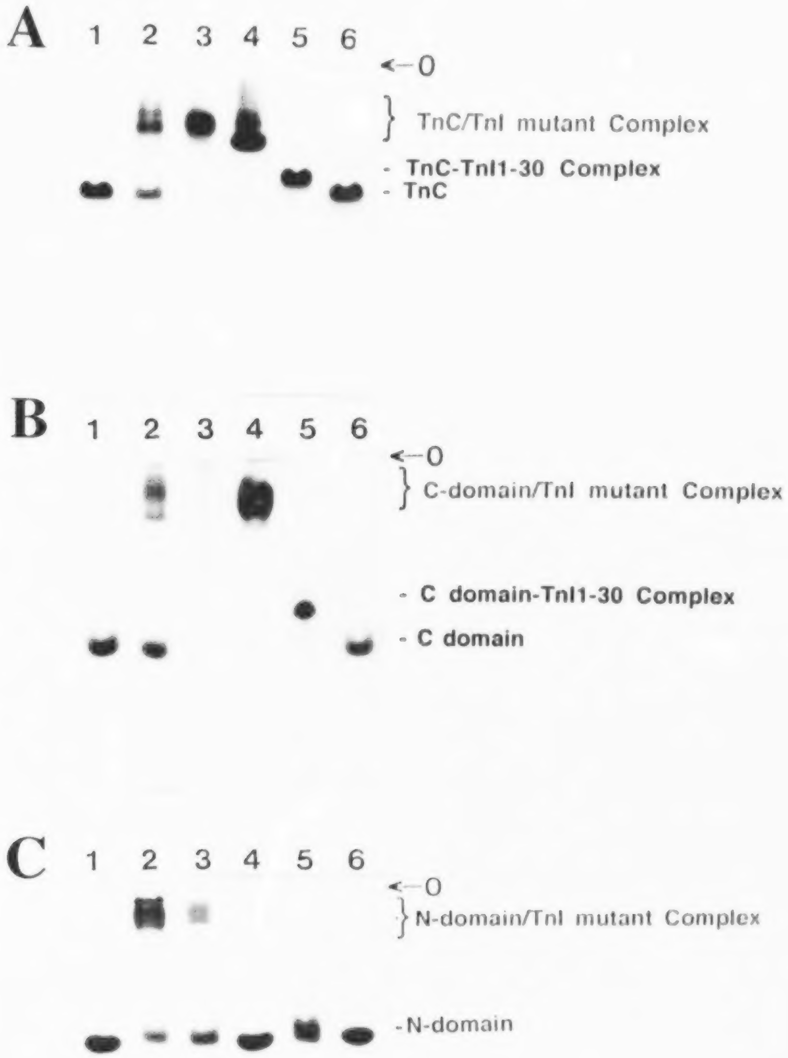
a. Native PAGE I (Complex Formation Study) - (Panel A) TnC/TnI analogs* Interaction : Lane 1, TnC (1 nmole); Lane 2, TnI (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 3, TnI$_{103-182}$ (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 4, TnI$_{1-116}$ (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 5, TnI1-30 (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 6, TnC (1 nmole). (Panel B) C-domain/TnI analogs Interaction : Lane 1, C-domain (1 nmole); Lane 2, TnI (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 3, TnI$_{103-182}$ (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 4, TnI$_{1-116}$ (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 5, TnI1-30 (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 6, C-domain (1 nmole). (Panel C) N-domain/TnI analogs Interaction : Lane 1, N-domain (1 nmole); Lane 2, TnI (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 hr; Lane 3, TnI$_{103-182}$ (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 hr; Lane 4, TnI$_{1-116}$ (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 hr; Lane 5, TnI1-30 (1.5 nmole) and N-domain (1 nmole) equilibrated for 1 hr; Lane 6, N-domain (1 nmole).

b. Native PAGE II (Competition Study) - (Panel A, left) TnI/TnC/TnI1-30 Interaction : Lane 1, TnC (1 nmole); Lane 2, TnI (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 3, TnI1-30 (2 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 4, TnI (1.5 nmole), TnC (1 nmole) and TnI1-30 (2 nmole) equilibrated for 1 hr; Lane 5, TnC (1 nmole). (Panel A, right) TnI/C-domain/TnI1-30 Interaction : Lane 1, C-domain (1 nmole); Lane 2, TnI (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 3, TnI1-30 (2 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 4, TnI (1.5 nmole) , C-domain (1 nmole) and TnI1-30 (2 nmole) equilibrated for 1 hr; Lane 5, C-domain (1 nmole). (Panel

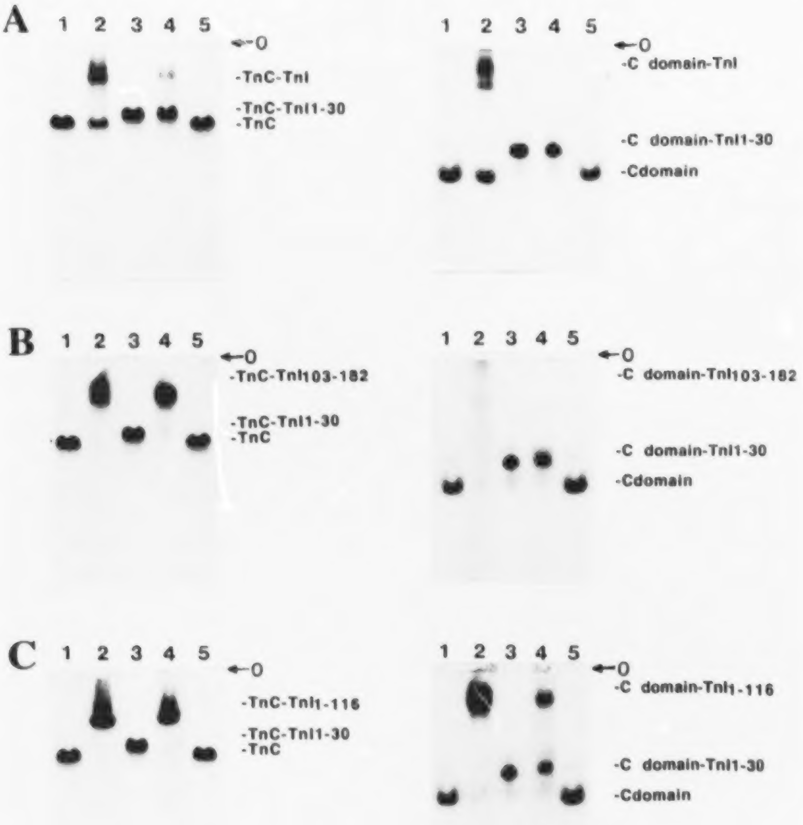
B, left) TnI$_{103-182}$/TnC/TnI1-30 Interaction : Lane 1, TnC (1 nmole); Lane 2, TnI$_{103-182}$ (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 3, TnI1-30 (2 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 4, TnI$_{103-182}$ (1.5 nmole), TnC (1 nmole) and TnI1-30 (2 nmole) equilibrated for 1 hr; Lane 5, TnC (1 nmole). (Panel B, right) TnI$_{103-182}$/C-domain/TnI1-30 Interaction : Lane 1, C-domain (1 nmole); Lane 2, TnI$_{103-182}$ (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 3, TnI1-30 (2 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 4, TnI$_{103-182}$ (1.5 nmole), C-domain (1 nmole) and TnI1-30 (2 nmole) equilibrated for 1 hr; Lane 5, C-domain (1 nmole). (Panel C, left) TnI$_{1-116}$/TnC/TnI1-30 Interaction : Lane 1, TnC (1 nmole); Lane 2, TnI$_{1-116}$ (1.5 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 3, TnI1-30 (2 nmole) and TnC (1 nmole) equilibrated for 1 hr; Lane 4, TnI$_{1-116}$ (1.5 nmole), TnC (1 nmole) and TnI1-30 (2 nmole) equilibrated for 1 hr; Lane 5, TnC (1 nmole). (Panel C, right) TnI$_{1-116}$/C-domain/TnI1-30 Interaction : Lane 1, C-domain (1 nmole); Lane 2, TnI$_{1-116}$ (1.5 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 3, TnI1-30 (2 nmole) and C-domain (1 nmole) equilibrated for 1 hr; Lane 4, TnI$_{1-116}$ (1.5 nmole), C-domain (1 nmole) and TnI1-30 (2 nmole) equilibrated for 1 hr; Lane 5, C-domain (1 nmole).

*TnI analogs do not enter gel.

a. Native PAGE I (Complex Formation Study)


A
1 2 3 4 5 6
<--O
TnC/TnI mutant Complex
- TnC-TnI1-30 Complex
- TnC
B
1 2 3 4 5 6
<--O
C-domain/TnI mutant Complex
- C domain-TnI1-30 Complex
- C domain
C
1 2 3 4 5 6
<--O
N-domain/TnI mutant Complex
-N-domain

b. Native PAGE II (Competition Study)


A
1 2 3 4 5
←O
-TnC-TnI
-TnC-TnI1-30
-TnC
1 2 3 4 5
←O
-C domain-TnI
-C domain-TnI1-30
-Cdomain
B
1 2 3 4 5
←O
-TnC-TnI103-182
-TnC-TnI1-30
-TnC
1 2 3 4 5
←O
-C domain-TnI103-182
-C domain-TnI1-30
-Cdomain
C
1 2 3 4 5
←O
-TnC-TnI1-116
-TnC-TnI1-30
-TnC
1 2 3 4 5
←O
-C domain-TnI1-116
-C domain-TnI1-30
-Cdomain

fact, C-domain/TnI$_{103-182}$ interaction is not as strong as C-domain-TnI1-30 interaction (lane 2 and 3 of Fig. VI-4b (panel B, right)). These verify the fact that TnI N- and C-domains flanking the inhibitory region interact in an antiparallel fashion with the corresponding N- and C-domain in TnC. Fig. VI-4b (panel C, right) shows that TnC/TnI$_{1-116}$ interaction is again stronger than the TnC/TnI1-30 interaction. In agreement with the above observation that deletion of the C-terminal region of TnI actually enhances the TnI/TnC interaction. In addition, TnI1-30 (residue 1-30) and TnI$_{1-116}$ (residue 1 - 116) are competing with the same binding site on TnC (both TnI$_{1-116}$ and TnI1-30 bind predominantly to C-domain (Ngai and Hodges, 1992 and Farrah *et al.*, 1994)). Fig. VI-4b, (panel C, right) indicates that both C-domain-TnI$_{1-116}$ and C-domain-TnI1-30 complexes can co-exist (lane 4). However, based on the intensity of the two bands corresponding to the two complexes (TnC C-domain/TnI$_{1-116}$ and TnC C-domain/TnI1-30 complexes) in lane 4, we can see that the C-domain-TnI1-30 complex prevails and this could be explained by the fact that the presence of the TnI sequence other than TnI1-30 together with the inhibitory region of TnI actually weaken the TnC C-domain/TnI$_{1-116}$ interaction. Nevertheless, their presence strengthens the TnC/TnI$_{1-116}$ interaction.

DISCUSSION

Previous investigations on TnI/TnC interactions demonstrated that two regions of TnI (residues 1-40 and 104-115) interact with TnC where three regions of TnC (residues 49-61, 89-100 and 127-138) can interact with TnI (Syska *et al.*, 1976; Weeks and Perry, 1978; Talbot and Hodges, 1979, 1981a, 1981b; Evans and Levine, 1980; Nozaki *et al.*, 1980; Grabarek *et al.*, 1981; Katayama & Nozaki, 1982; Leavis and Gergely, 1984, review; Van Eyk and Hodges, 1988). Wang *et al.* (1990) demonstrated that binding of cations (Ca^{2+} or Mg^{2+}) to the high affinity sites (III and IV) of a mutant TnC altered the environment around the amino acid at position 57 in the N-terminal domain (Sites I and II).

Grabarek *et al.* (1986) and Rosenfeld and Taylor (1985) showed that the binding of Ca^{2+} to the low affinity sites (I and II) altered the environment around Cys 98 in the C-domain of TnC. TnC in the presence of TnI adopts a more compact conformation in solution (Wang *et al.*, 1987) than in the crystal structure of TnC (Sundaralingam *et al.*, 1985; Herzberg and James, 1985 and 1988). Studies have indicated that the TnI inhibitory region (residues 104-115 (Ip) or a longer version of Ip (residues 96-116)) can interact with both the C-terminal domain of TnC (Weeks and Perry, 1978; Chong and Hodges, 1981, 1982a,b; Leavis and Gergely, 1984; Dalgarno *et al.*, 1982; Drabikowski *et al.*, 1985; Tao *et al*, 1986; Leszyk *et al.*, 1987,1988; Lan *et al.*, 1989; Van Eyk *et al.*, 1991; Swenson and Fredricksen, 1992) and N-terminal domain of TnC (Leszyk *et al.*, 1990 and Kobayashi *et al.*, 1991). The above observations suggested that the TnI inhibitory region may form a single binding site between the N- and C-domain of TnC. This is supported by our results in which intact D/E helix of TnC is important in maintaining the full biological function of TnC in interacting with the TnI inhibitory peptide, since neither TnC C-domain nor TnC N-domain were able to fully release the Ip inhibition of the acto-S1-TM ATPase activity (Fig. VI-1). Our results is comparable with earlier work using proteolytic fragments of TnC on the studies of TnC/TnI interaction (Grabarek *et al.*, 1981). Nevertheless, our studies together with the above investigations have suggested that the major TnC binding sites for the inhibitory region of TnI is located on the C-domain of TnC and we have proposed a computer generated three-dimensional structure of the TnC C-domain/Ip complex (Ngai *et al.*, 1994).

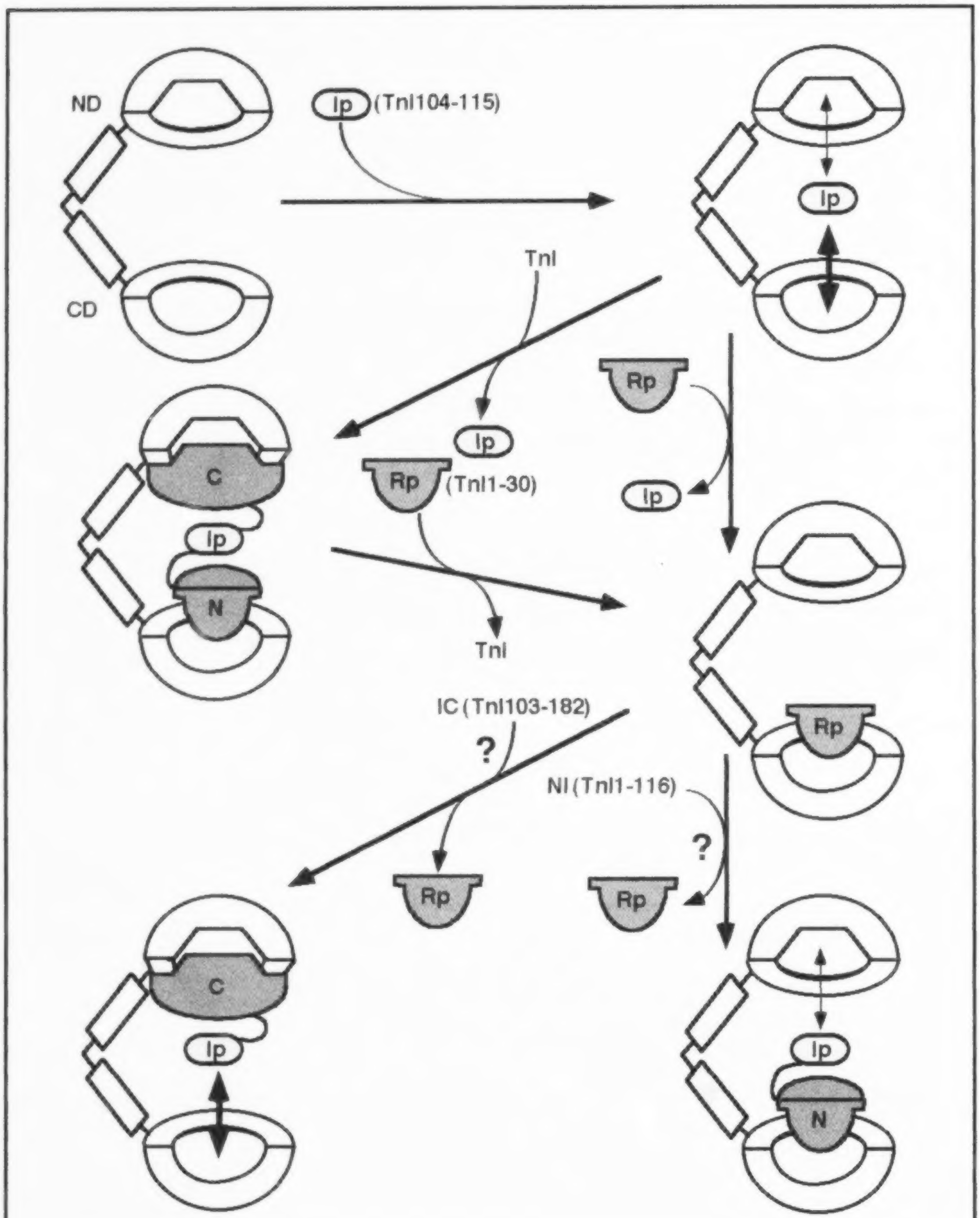
Very little attention has been given to the biologically important interaction between TnC and the N-terminal region of TnI since it was first identified by Syska *et al.* (1976). In our previous investigation, we have shown that synthetic peptides corresponding to the N-terminus of TnI were able to interact with TnC and prevent TnC from neutralizing TnI or TnI inhibitory peptide (Ip) induced inhibition of acto-S1-TM ATPase activity. This raises the question of how the N-terminal region of TnI governs the release of the inhibitory region of TnI within the TnI/TnC complex and be fitted into the Ca^{2+}-sensitive control of muscle contraction and relaxation event.

Perry *et al.* (1975) had proposed two mechanisms to describe how troponin C may interact with TnI to neutralize the inhibitory activity of TnI. First it was postulated that the two sites (referred to as TnI1-30 (TnI residues 1-30) and Ip (TnI residues 104-115) in this study) are located on the troponin I molecule so that the binding of one site with TnC takes place without physical obstruction of the other. In this case, the interaction of TnC at the N-terminal region of TnI in the presence leads to a conformational change that causes modification in the molecule in the region of Ip so that the Ip site is no longer available for interaction with actin. Though this scheme did not propose the strong interaction of TnC with the inhibitory peptide Ip in the presence of Ca^{2+}(Van Eyk & Hodges, 1988), this interaction is compatible with Perry's proposal. However, the large conformational changes in TnI were ruled out by studies showing that Ca^{2+} induced changes in the TnC/TnI complex were only slightly greater than the sum of those in the separate subunits as measured by circular dichroism (McCubbin *et al.*, 1973).

The second scheme requires that regions of troponin I (Rp and Ip) that interact with TnC and actin are located close together on the surface of the TnI molecule so that Ca^{2+}-induced interaction with TnC effectively prevents actin from interacting with the inhibitory region (Ip). Compatible with this proposal is that TnI Ip region is able to bind to actin and inhibit the actomyosin ATPase activity and bind to TnC causing the release of the ATPase inhibition (Talbot and Hodges, 1979, 1981a, 1981b; Katayama and Nozaki, 1982; Cachia *et al.*, 1983, 1986; Van Eyk and Hodges, 1987, 1988). It has been proposed that one of the chemical switches in muscle regulation involves the binding of Ip to actin preventing the S1-actin interaction (muscle relaxation). In the presence of Ca^{2+}, conformational changes in TnC result in the release of inhibition via TnC binding to the Ip region, thus allowing the interaction of S1 and actin (muscle contraction).

Binding of TnI1-30 to TnC was shown to prevent the ability of TnC to interact with the inhibitory region, Ip in neutralizing the inhibition of the acto-S1-TM ATPase activity caused by Ip (Ngai and Hodges, 1992). Adding to our previous findings are : 1) TnI N-terminal region interacts predominantly with the C-domain of TnC. 2) It can interact with TnC in its apo, Mg^{2+} or Ca^{2+} states (Ngai *et al.*, unpublished results) and upon the binding of TnI1-30 to TnC, there is an increase in Ca^{2+}- affinity at the high affinity sites (sites III & IV) of the TnC protein. 3) The increasing orders of the strength of interaction between TnC and TnI analogs are Ip/TnC < TnI/TnC < TnI1-30/TnC < (TnI$_{103-182}$/TnC, TnI$_{1-116}$/TnC) respectively. Our studies on the TnI/TnC interaction are summarized in Fig. VI-5. This model agrees with results from two other recent investigations on the biological function of the TnI N-terminus (Sheng *et al.*, 1992 and Farrah *et al.*, 1994) as well as our previous studies of the biological function of the TnI N-terminal regulatory peptide (residues 1-40).

Fig. VI-5. **Summary for TnI/TnC Interaction.** TnC molecule is represented by a cross-section of the curved dump bell figure and ND represents N-domain and CD represents C-domain of TnC; N, TnI residues 1 to 98 (red); Ip (yellow), TnI residues 104 to 115; C, TnI residues 120 to 182 (red); TnI, TnI residues 1 to 182; Rp (blue), TnI residues 1 to 30; $\text{TnI}_{1\text{-}116}$, TnI residues 1 to 116; $\text{TnI}_{103\text{-}182}$, TnI residues 103 to 182. Thick double-headed arrow represents strong interaction and thin double-headed arrow represents weaker interaction (see text for detail).



ND
CD
Ip
(TnI104-115)
Ip
TnI
Rp
Ip
C
Rp
(TnI1-30)
Ip
Ip
N
TnI
IC (TnI103-182)
?
Rp
NI (TnI1-116)
?
Rp
Rp
C
Ip
Ip
N

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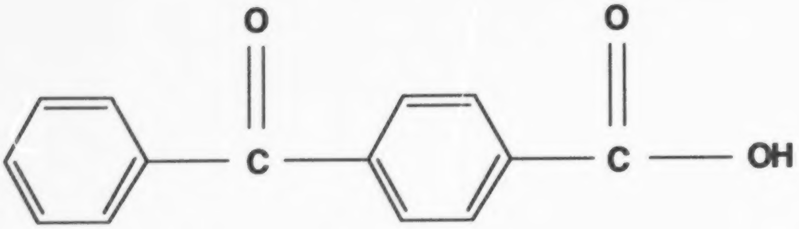
CHAPTER VII

DISCUSSION AND FUTURE RESEARCH

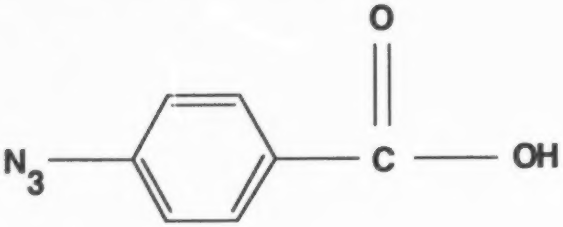
One promising approach to the studies of protein/protein interactions involved in muscle regulation is to carry out specific cross-linking experiments on various components of the thin filament, which define their interactive relationships within the complex. Early in the fifties, chemical cross-linking techniques had been widely used in protein structure elucidation and as affinity labeling in the sixties to determine the amino acid residues involved within the binding interface between proteins (Wold, 1972). The theory of affinity labeling depends on the fact that the formation of most biological complexes involves a number of favorable interactions that make up the ligand-receptor recognition process. The total binding free energy upon complex formation is usually negative enough to allow minor modification of the structure of the native ligand without significantly decreasing either the selectivity or the strength of the overall binding. A typical method of affinity labeling involves incorporation of a chemically labile functional group into the ligand and cross-linking the modified ligand to the binding site through a chemical reaction (Wold, 1972). Information obtained from these crosslinking experiments has enabled the use of a synthetic peptide approach to define the structural and functional relationship within the regulatory complex.

Although there were successes in using such labile functional groups for labeling the binding interface between proteins, there are several limitations to this type of approach: a) The functional groups may not be stable in aqueous solution at physiological pH (7-9). For example, the imidoester has a half life of about 10 min. at 4 °C in aqueous solution. b) There is no control of the chemical reactivity of the labile groups. c) Reactivity of the functional groups depend on the appropriate condition of pH, temperature and the environment of the binding interface where the probe is located, so it is difficult to optimize the cross-linking conditions under various conditions required for protein-protein binding. d) Successful chemical cross-linking depends on the availability of suitable reactive groups (e.g. Cys, Lys, Arg, His, Glu and Asp) that are located within the effective range for reaction with the probe. In order to solve the above problems, various photoaffinity probes (p-azidobenzoic acid (AB), benzoylbenzoic acid and p-nitrophenylalanine) were introduced (Galardy *et al.*, 1974; Escher et al., 1982; Staros, 1980). Photoprobes (Fig. VII-1) have the property of being inert until photolysis and are nonspecific in coupling to the target protein. The photoactivated species will react with primary, secondary and tertiary carbons along with various functional side chains of a protein. Benzophenone is stable in glacial acetic acid or aqueous solution. Also, the benzophenone and p-nitrophenylalanine probes are stable to the hydrofluoric acid conditions used in deprotection and cleavage of the peptide from the resin support in solid-phase sythesis. An obvious advantage of these two probes is that they can be coupled directly to a protected peptide on the resin using the same conditions employed for amino acid couplings. It is important to note that this route does not need any specific functional group to link the probe to the peptide and eliminates any modification in the sequence of interest (Parker and Hodges (1985a, b).

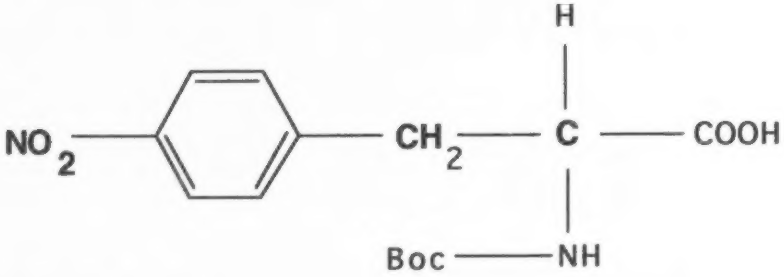
In Chapter III of this thesis, we have described the application of a photoaffinity probe, BB (benzoylbenzoic acid) to the study of the Ca^{2+}-sensitive TnI/TnC interaction. The photoactivatable benzoylbenzoyl probe (BB) was attached to the N-terminus of the TnI inhibitory peptide, Ip (residues 104-115) through standard solid-phase methodology. The resulting peptide (BBIp) retains full biological activity. BBIp is capable of interacting with TnC and inhibiting the acto-S1-TM ATPase activity. The crosslinking results indicated that the binding of BBIp to TnC is specific in either Mg^{2+} or Ca^{2+} buffer (Chapter III). Through characterization of the crosslinked TnC/BBIp complex, we are able to locate the crosslinking site to a single residue (Met-155) of the TnC protein. Our results are consistent with other investigations (see Chapter III). A three-dimensional model of the TnC C-domain/Ip complex was generated using molecular dynamic and energy minimization



BB



AB



p-NO_2 -Phe-Ala

Fig. VII-1. **Structure of photoaffinity probes used to prepare synthetic probe-peptides**. BB, p-benzoylbenzoic acid; AB, p-Azidobenzoic acid; p-NO_2-Phe-Ala, p-nitrophenylalanine.

calculations (the TnC structure has been determined previously by crystallography and the Ip bound structure by NMR) and we concluded that the major Ip binding site was located on the hydrophobic groove of the C-domain of TnC. These crosslinking results have demonstrated the feasibility of using potoaffinity labelling of synthetic peptide to locate the sites of interaction between other members of the regulatory protein complex. On-going photoaffinity labeling experiments involves the use of BB on the study of the interaction between TnC and N-terminal peptide of TnI. We have synthesized a TnI N-terminal peptide (Residues 1-40) with the BB moiety attached through the N-terminus and we have crosslinked it to TnC. Characterization of the crosslinked TnC/BB-peptide will assist us in understanding the TnI/TnC interaction.

In Chapter IV and V, we demonstrated that TnI N-terminal peptides [TnI1-40, Rp (residues 1-40) and TnI1-30 (residues 1-30)] are able to prevent TnI or Ip from interacting with TnC. Fluorescence titration of TnI1-30 with TnC mutants that carry a single fluorescence probe in either the N- or C-domain [F105W, F105W/C domain (88-162), F29W and F29W/N domain (1-90)] demonstrates low Kd values for both F105W mutant and F105W/C domain (88-162) ($Kd < 10^{-7}$ M) towards binding of TnI1-30. However, there is no observable change in fluorescence if the fluorescence probe is located at the N-domain of the TnC mutant [F29W and F29W/N domain (1-90)]. Photochemical crosslinking experiments together with gel electrophoresis studies indicated that TnI1-30 can interact with TnC in its apo, Mg^{2+} or Ca^{2+} states and it has been shown using benign gel electrophoresis that TnI1-30 is only capable of forming a stable complex with either TnC or TnC C domain in the presence of Ca^{2+}. The above results suggest that TnI N-terminal peptide is always capable of interacting with the C-domain of TnC. Binding of TnI N-terminal peptide to TnC will prevent Ip and intact TnI from interacting with TnC.

In Chapter VI, the use of both TnC N-terminal (residues 1-90) and C-terminal domains (residues 88-162) demonstrated that an intact central helix (D/E helix) linking the two domains of TnC was important in retaining its full biological function in interacting with the TnI inhibitory region (residues 104-115). Circular dichroism studies indicated that the Ca^{2+}-affinity of the C-domain of TnC was enhanced upon the binding of TnI1-30 to TnC. The TnC/TnI1-30 and C domain/TnI1-30 complexes isolated by size-exclusion chromatography (SEC) showed that TnI1-30 forms a 1:1 protein : peptide complex. By using deletion mutants of TnI in conjunction with TnI1-30 and recombinant TnC mutants binding studies using native gel electrophoresis have indicated that N- and C-terminal regions of TnI interact in an antiparellel fashion with the corresponding N-domain and C-domain of TnC. These results are consistent with two other recent investigations on the TnI/TnC interaction (Sheng *et al.*, 1992; Farrah *et al.*, 1994). On the basis of our present results, we have proposed a modified schematic diagram for the TnI/TnC interaction in the regulation of muscle contraction in Fig. VII-2. Investigations from our laboratory using synthetic peptides have shown that the regions of actin, residues 1-28, and TnI, residues 104-115, are two components of the Ca^{2+}-dependent switch that controls muscle relaxation and contraction. The TnI inhibitory peptide, which interacts with actin-TM causing inhibition of the S1 ATPase activity, also binds to TnC. As well, the actin peptide, which interacts with S1 (and HMM) causing activation of the ATPase activity, also binds to the inhibitory region of TnI (Fig. VII-2). However, our studies on the N-terminal region of TnI definitely demonstrate that this region of TnI also takes part in the regulatory process. In the new model, we propose that in the absence of Ca^{2+} (when either Mg^{2+} or Ca^{2+} is bound to the two high-affinity Ca^{2+} binding sites III and IV of TnC), the N-terminal region of TnI is able to interact with the C-domain of TnC. This interaction prevents Ip from interacting with TnC. The interaction between the TnI inhibitory region and actin is dominant (Fig. VII-2). Muscle relaxation is then promoted due to inhibition of the actin and myosin interaction and thus the ATPase activity (Van Eyk *et al.*, 1986). In the presence of Ca^{2+} (when Ca^{2+} binds to the low-affinity Ca^{2+} binding sites I and II of TnC), interaction

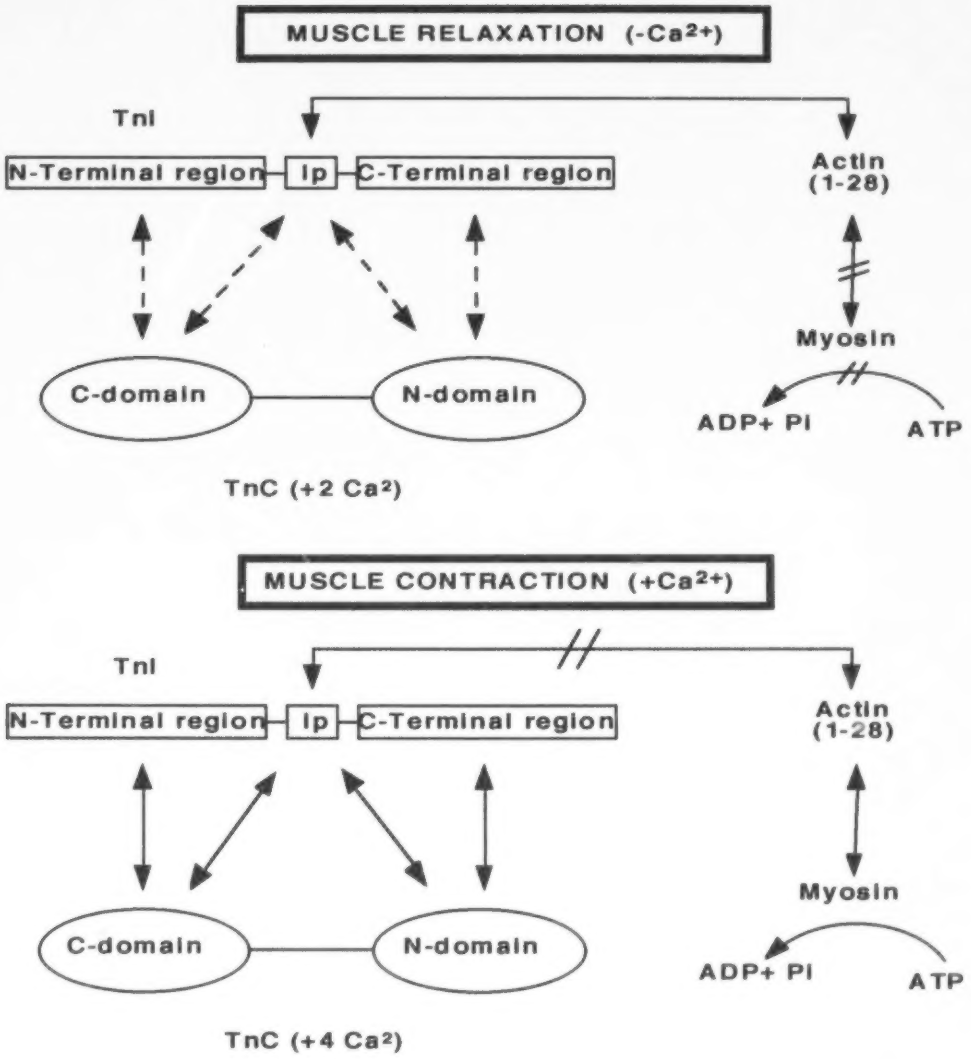

MUSCLE RELAXATION (-Ca^{2+})
TnI
N-Terminal region
Ip
C-Terminal region
Actin (1-28)
Myosin
ADP+ PI
ATP
C-domain
N-domain
TnC (+2 Ca^{2})
MUSCLE CONTRACTION (+Ca^{2+})
TnI
N-Terminal region
Ip
C-Terminal region
Actin (1-28)
Myosin
ADP+ PI
ATP
C-domain
N-domain
TnC (+4 Ca^{2})

Fig. VII-2. **Schematic diagram showing the dominant protein interactions involved in the regulation of muscle contraction**. Panel A shows the dominant interactions when the Ca^{2+} specific sites I and II are unoccupied and muscle is relaxed. Panel B shows the dominant interactions when the Ca^{2+} specific sites I and II are occupied and muscle is contracted. Solid lines represent strong interactions whereas broken lines represent weak interactions.

between Ip together with C-terminal region of TnI and the N-domain of TnC is strengthened, the N-terminal region of TnI is now no longer capable of preventing the Ip region of TnI from interacting with TnC. TnI inhibition is released since the TnI inhibitory region (residues 104-115) binds to TnC (Van Eyk and Hodges, 1988) and actin 1-28 can no longer bind Ip (Van Eyk, *et al.*, 1991). Actin 1-28 then interacts with myosin, causing the activation of the ATPase activity and muscle contraction. This model shows only the TnI/TnC interactions that were the subject of this thesis. TnT and TM both play important roles in regulation of muscle contraction. It is well documented that the presence of TM in the ATPase assay greatly enhances the activity of TnI and TnI peptide 104-115 (Talbot and Hodges, 1979, Eaton *et al.*, 1975). TnT is also involved in the interaction with the N-terminal region of TnI (Hitchcock et at, 1981; Sheng *et al.*, 1992; Chong and Hodges, 1982 and Farah *et al.*, 1994).

On going research involves NMR studies on the interaction of TnC and TnI N-terminal peptide (residues 1-30). Preliminary data demonstrates that there are significant chemical shifts on the residues within the E, F, G and H helices of the TnC C-domain upon binding of the TnI1-30 peptide (Calhoun *et al.*, unpublished results). NMR studies on the TnC/TnI1-30 complex will yield information on the bound structure of TnI1-30 peptide. In addition, the structure of the TnI inhibitory peptide (Ip) bound to TM-actin complex is important to allow comparison of the structure of the peptide bound to TnC. Research has just begun using TnI N-terminal peptide as a probe to understand regulation of muscle contraction. Studies of this nature shall have the potential for probing details of the interactions of the thin filament components that are dependent upon the presence of all the subunits.

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